AN ANALYSIS OF ALTERNATIVE FORMS OF PLANT REPRODUCTION USING *GOSSYPIUM BARBADENSE* AND *ARABIDOPSIS THALIANA*

A Dissertation

by

KELLY DENISE BIDDLE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2006

Major Subject: Molecular and Environmental Plant Sciences

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Approved by:

Chair of Committee,	David Stelly
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ABSTRACT

An Analysis of Alternative Forms of Plant Reproduction Using *Gossypium barbadense* and *Arabidopsis thaliana.* (December 2006)

Kelly Denise Biddle, B.A., Rice University

Chair of Advisory Committee: Dr. David Stelly

Apomixis holds vast potential for improving agriculture worldwide. It would make plant breeding faster, allow for fixation of hybrid genotypes, and help genetically isolate transgenic crops. However, efforts to introduce apomixis into agriculturally important crops, through either plant breeding or molecular genetics, have failed to produce any new apomictic varieties. This could possibly be remedied if researchers better understood the genetics of apomixis, including the underlying genes, their regulation, and the cellular pathways they control. My work increased our knowledge of these processes by using a mixture of novel methods and underutilized resources.

In this study I pursued a two-pronged approach that involved both traditional and reverse molecular genetic techniques. I analyzed the *Semigamy* mutation from Pima Cotton (*Gossypium barbadense*), which produces large numbers of haploid and chimeric offspring. Although interesting and potentially useful, little is known about this gene, its expression, regulation, and localization, or the cellular processes in which it is involved. This knowledge advanced our understanding of both this part of apomixis and plant reproduction in general. I was able to determine that the *Semigamy* mutation is recessive to the wild-type and is incompletely expressed even in the homozygous state.

Second, I attempted to recreate the various steps of apomixis in the model species *Arabidopsis thaliana* using the host of tools made available by its fully sequenced genome. Mutants in various organisms, including microbes, plants, and animals, have phenotypes resembling various components of apomixis, and the sequences of most of the genes involved are available in public databases. I identified homologous *Arabidopsis* genes by comparing these sequences against the entire *Arabidopsis* genome. Mutants carrying altered versions of these genes were then studied and characterized using various techniques to see if they had any effect on plant reproduction. This method had not been used to study apomixis before this study and revealed several new reproductive *Arabidopsis* mutations, specifically those in genes homologous to the *S. cerevisiae ste12* and *rec8* genes. These results will advance the study of apomixis and potentially enable researchers to one day apply it to agriculturally important crop species.

DEDICATION

To my family:

Mom, Dad, Robert, and Wesley

And to my angels:

Mamaw Biddle, Papaw Biddle, and Granny Klein

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I would like to thank all of my co-workers for the past four years. Though at times they have driven me insane, they always kept me from completely losing it. I especially want to thank Wayne Raska for letting me use his aneuploids and for keeping my plants alive, in spite of my best efforts otherwise. I also need to thank his army of student workers that have helped us all in the fields, in the greenhouses, and in the lab. Thanks also to Brian Gardunia, Nilesh Dighe, Steve Todd, Naomi Schechter-Waak, Christian Hans and many other graduate students who became close friends and confidants. We had some fun times.

I would also like to thank Dr. Stelly for his guidance over the years. He was the one to first suggest this project, and he was always the first person I went to with problems and conundrums. He was also willing to review all my presentations and manuscripts and provide many helpful comments. I would also like to thank George Hodnett for his helpful guidance. Much of this research began with George, who taught me all the initial microscope techniques. George was also always a willing ear for my problems and confusion before I was ready to talk to Dr. Stelly.

I would also like to thank my committee for their guidance and the very interesting discussions we have had. Dr. Chen allowed me into his lab to conduct much of the *Arabidopsis* molecular genetics work, and Dr. McKnight advised me on how to conduct the tricky *in silico* BLAST searches. Dr. Polymenis provided invaluable insight into yeast and chromosome biology. These gentlemen were always wonderfully helpful, and I could not have graduated without them.

Though this dissertation is dedicated to my immediate family, I have been blessed with a large, supportive family of grandparents, aunts, uncles, cousins, in-laws, and more. I could never have done any of this without their love, support, and encouragement. They are a part of me and all that I am.

Lastly, I offer unconditional gratitude to God, who strengthens, guides, and loves me always. Jesus looked at them and said, "With man this is impossible, but with God all things are possible." (Matthew 19:26)

I will praise you, O LORD, with all my heart;
I will tell of all your wonders.

I will be glad and rejoice in you;
I will sing praise to your name, O Most High. (Psalm 9:1-2)

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INTRODUCTION

The study of apomixis, or asexual reproduction through seed, is a rapidly growing field with far-reaching implications in plant breeding, biotechnology, and world food production. Unfortunately, characterizing apomictic reproduction and analyzing its genetic causes has been hampered by the wide diversity of apomictic mechanisms, the absence of an universally accepted "model species" for the study of apomixis, and the paucity of genetic information from the species in which it is well-studied. Although the benefits of generating apomixis in agriculturally important crop species are well-documented (Koltunow et al. 1995), achievement of such goals will remain difficult until scientists better understand of the developmental mechanisms, molecular components, and genetic regulatory systems underlying both sexual and asexual plant reproduction.

Mechanisms of Apomixis

In most forms of sexual plant reproduction, a megaspore mother cell undergoes meiosis to produce four haploid cells. Three of these cells degenerate while one, the megaspore, begins to enlarge and develop. The megaspore undergoes three rounds of endomitosis, resulting in one large syncytium containing eight nuclei. This immature 8-nucleate megagametophyte undergoes regulated cytokinesis to form the seven separate cells that compose the embryo sac: three antipodals of unknown function, two synergids that support the egg cell and guide pollen tube growth, a central cell containing two

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This dissertation follows the style of Plant Cell.

polar nuclei that will eventually fuse with a pollen nucleus to form the endosperm tissue that nurtures the developing embryo, and one large, vacuolate egg cell. This mature 7-cell, 8-nucleate megagametopyte and the surrounding diploid maternal tissue form the nucellus (Schneitz et al. 1997). During fertilization, a pollen tube penetrates the egg sac via the filiform apparatus and releases two haploid sperm cells. One sperm cell fuses with the central cell, permitting a sperm nucleus to fuse with the two polar nuclei and form the triploid endosperm. The second sperm cell unites with the egg cell, and its nucleus fuses with the egg nucleus to create the diploid zygote (Faure et al. 2002).

Recurrent apomictic reproduction differs from sexual reproduction in three key elements: the retention of the somatic chromosome number in the reproductive cell, prevention of maternal and paternal genomic fusion, and autonomous development of the unreduced cell into a viable embryo (Koltunow and Grossniklaus 2003). The first is necessary to prevent the formation of haploid offspring that inherit only the maternal genome, while the second prevents the formation of offspring with ever-excalating ploidy. Progeny produced through the fertilization of unreduced egg cells would be one ploidy level higher than their maternal parent, and if this trend were continued over several generations the resulting abnormally large and highly duplicated genomes would potentially hamper plant fertility and viability. Lastly, in most sexual species unfertilized egg cells normally degenerate relatively quickly, so some mechanism must allow apomictic cells to continue developing in the absence of the fertilization stimulus.

Most mechanisms for generating diploid reproductive cells during recurrent apomixis are generally grouped into the distinct categories: adventitious embryony,

diplospory, and apospory. The latter two are commonly grouped under the subheading "gametophytic apomixis" because they depend on the generation of embryo sacs similar to those seen in sexual reproduction (Savidan 2000). Adventitious embryony, however, is the generation of embryos directly from the somatic tissues in the ovary of the plant (Koltunow et al. 1995). Because of this, it is also known as sporophytic apomixis (Savidan 2000). Adventitious embryony typically occurs several times within one sexually fertilized ovule. In some cases one of the apomictically-derived embryos invades the reduced embryo sac and derives its energy and nutrients from the sexually derived endosperm. In other instances several embryos can grow and develop concurrently, resulting in polyembryonic seed (Koltunow 1995; Asker and Jerling, 1992). Though well documented in citrus, this apomictic mechanism is uncommon in other populations and has not been studied extensively in the lab. Instead, most researchers choose focus on aposporous or diplosporous reproduction.

Apospory involves the formation of embryo sacs from the nucellar cells that surround the meiotically produced and reduced embryo sac. The aposporous embryo sac can either be eight-nucleate (*Hieracium* type—one egg cell, two synergid cells, three antipodal cells, and two polar nuclei in the central cell) or four-nucleate (*Panicum* type—one egg cell, two synergid cells, and one central nucleus in the central cell). Both of these forms occur naturally and with relatively high frequencies in wild apomictic populations (Crane 2001), though there does seem to be some form of climatic regulation. The *Hieracium*-type is seen more in temperate grasses while the *Panicum*-type dominates in tropical and subtropical regions (Savidan 2000). However, the overall

outcome of both modes remains the same—the unreduced egg cell of the aposporous egg sac grows and divides until it develops into a viable embryo. Often apospory occurs simultaneously with normal sexual gametogenesis, fertilization, and development; it can also occur several times within one ovule. This results in polyembryonic seed containing one sexually derived embryo and several aposporous and maternally clonal sister embryos.

In contrast, diplospory results from direct interference with megasporogenesis. Diplosporous plants either forgo meiosis completely or undergo an altered form that fails to reduce the chromosome number of the resulting megaspore (apomeiosis) (Savidan 2000). As in apospory, there are several different types. In the rare *Allium* type an endoduplication event occurs immediately before meiosis. Therefore, although meiosis occurs normally its products retain the somatic chromosome number (Savidan 2000). In the *Taraxacum* type of diplospory, the chromosomes fail to pair and there is no first division. A restitution nucleus is formed instead, which then divides to produce a dyad of unreduced cells. One cell from this pair will degenerate, and the other will undergo megagametogenesis. Lastly, in the most common form of diplospory, the *Antennaria*-type, meiosis is entirely omitted and the MMC divides mitotically three times to produce the embryo sac. Each nucleus in this embryo sac has exactly the same genetic constitution as the maternal tissues (Asker and Jerling 1992).

Just as there are multiple mechanisms for generating unreduced reproductive cells, plants have also evolved several different means to prevent fertilization of apomictically derived ovules. Although these mechanisms are not as extensively

characterized as those given above are, some of the details are beginning to become known. One in particular, semigamy, is defined as when the sperm nucleus enters the egg cell (plasmogamy or fertilization) but fusion of egg and sperm nuclei (syngamy or kayogamy) does not occur (Battaglia 1954). This reproductive abnormality has been discovered and characterized in a wide variety of both sexual and apomictic plant species, including *Cooperia pedunculata* (Coe 1953) and various *Rudbeckia* species (Battaglia 1954). In the apomictic species *Zephranthes pulchella* semigamy results in the sperm nuclei being sequestered into a small, developmentally terminal part of the egg cell; it does not contribute to the zygote (Crane 1978). It is assumed that in semigamous species the sperm nucleus only functions to initiate divisions in the unreduced egg cell (Turcotte and Feaster 1974).

Timing of embryo development also serves as a second mode of preventing parental genomic mixing. In aposporous apomicts the relative timing of apomictic and sexual development greatly influences the final outcome (Nogler 1984). In general, aposporous embryo sacs are initiated much earlier in development, so they are entirely matured by the time the pollen tube penetrates the ovule. Specifically, Savidan (1982) showed that in *Panicum maximum* aposporous and sexual embryo sacs can be statistically differentiated based solely on the timing of development. The vast majority of aposporous embryo sacs were past their receptive stage by the time the pollen tube reached the ovule, and some had even begun mitotic divisions. These results are also supported by classical observations that delayed pollination of sexual ovules induces haploid formation (Kimber and Riley 1963). Also, in 1994 Martinez found that early

pollination of apomictic plants increases the occurrence of triploid (2n + n) progeny, which also supports this theory that the production of unfertilized, unreduced embryos is temporally regulated.

The final requirement for apomixis is autonomous development of the doubled reproductive cell. However, apomixis is not synonymous with parthenogenesis. While some species, *e.g. Hieracium,* can develop in the complete absence of pollination, this is generally relegated to a very small taxonomical group. In most apomictic species even though the embryo is formed parthenogenetically its development and viability are dependent upon fertilization in some way. This makes these species pseudogamous, that is, their reproduction requires pollination without the effects of egg cell fertilization. Most pseudogamous species require fertilization to initiate endosperm development; however, many of the sexual regulations concerning endosperm fertilization are often absent. In most sexual species a 2 maternal : 1 paternal endosperm genomic ratio is required for seed development and viability (Lin 1984). However, most apomicts have a 4 maternal : 1 paternal ratio (2 fused, unreduced polar nuclei : 1 reduced sperm nucleus). When researchers attempt to mimic this unique combination through tetraploid x diploid crosses, the offspring are usually abnormally developed or sterile (Birchler 1993; Grimanelli 1997). In contrast, some species, such as *Panicum*, avoid the problem entirely by simply allowing only one maternal, unreduced polar nucleus to fuse with the sperm nucleus (thereby generating the standard 2m : 1p ratio). In *Ranunculus auricomus* two unreduced polar nuclei fuse with *two* reduced sperm nuclei, which generates a 6x endosperm with the required 2m : 1p final ratio (Savidan 2000). As with every other

part of apomixis, the mechanisms involved in endosperm fertilization are many and varied.

It is important to note that none of the above mechanisms completely eliminates sexual reproduction. Apospory and adventitious embryony occur alongside sexual embryo sac development. Successful diplosporous embryo sac formation can be influenced by several different environmental factors including temperature, humidity, and light levels, so diplosporous individuals often produce a mixture of reduced and unreduced embryo sacs, and the ratios between the two categories can vary widely. Additionally, it has been shown that semigamy is also environmentally regulated, and developmental timing often results in overlap between aposporous and sexual development. In short, none of these mechanisms is foolproof; they simply influence the overall ratios of apomictic to sexual offspring. Therefore, it is not surprising that the vast majority of apomictic species identified to date are facultative apomicts that are capable of both sexual and asexual reproduction within the same plant. In contrast, early research efforts identified most apomictic species as being obligate, or producing only maternally clonal offspring. It is now thought that these identifications may be artifacts of older, less accurate, and less thorough methods of screening and evaluation. Newer techniques, such as whole-ovule clearings (Hodnett 1997), larger population sizes, and more generations available for analysis, have allowed researchers to better ascertain the exact modes and mechanisms of apomictic development and to detect the small percentage of plants that avoid the asexual pathway. For example, in 1973 Burton found 80% obligate apomicts among accessions of *Panicum maxicum*, but a decade later

Savidan (1982) reviewed the same material using 10-plant progeny tests of 100 cleared ovules per accession and found only 20% of the population was obligate (Savidan 2000).

Apomixis has evolved naturally in several diverse angiosperm lineages, including at least 300 species in 35 families (Koltunow et al. 1995), and it never seems to evolve the same way twice. The wide degree of physical variability among all the different forms and systems of apomixis has kept the study of this phenomenon both interesting and confusing. It also implies the genetic mechanisms that condition, initiate, and regulate apomictic development, once elucidated, will be just as varied.

Genetic Regulation of Apomixis

While the physical aspects of apomixis have been studied for nearly a century, the genetics behind its control and regulation have been left mainly in the dark. Classical Mendelian studies were hampered by the limited sexuality of these species. However, with the advent of new tools in molecular biology and genetics researchers have begun to unravel some of the specific genes and signaling/interaction pathways that control both apomictic and sexual reproduction.

Gian Nogler performed pioneering genetic work with *Ranunculus* that showed that apomictic reproduction segregates as a dominant trait. He made crosses between closely related apomictic and sexual species and traced the sexuality of the initial and backcrossed progeny for several generations. His results indicated that in this species one dominant allele (A-) controls both parthenogenesis and fertilization-independent endosperm development. Hence aposporous apomixis can be inherited as a single

genetic trait (Nogler 1984; Grossniklaus. 2001). Apomictic individuals must have one or more copies of the A- allele, but, interestingly, no homozygous A- plants have ever been identified. Apparently the wild type a+ allele is necessary for plant viability and/or reproductive potential. Additionally, the A- allele cannot be transmitted by haploid gametes. It is highly probable that A- acts as a recessive lethal (Nogler 1984). These results help to explain the often observed relationship between polyploidy and apomixis (Grossniklaus et al. 2001; Quarin et al. 2001)—the higher the level of ploidy, the more protected a species is against homozygous A- formation. Using similar methods, several other researchers have supported Nogler's initial findings. Within several members of the *Hieracium* genus (e.g. *aurantiacum* and *piloselloides*) apospory segregates as one dominant gene (Gadella 1991; Koltunow et al. 1995; Bicknell 1997). Dominant segregation has been documented in other genera as well—*Panicum, Brachiaria,* and *Pennisetum* (Bicknell et al. 2000, Sherwood et al. 1994, Valle et al. 1994)—and diplospory in *Tripsacum dactyloides* acts as a dominant trait (Leblanc et al. 1995).

However, the results of several recent studies have begun to contradict this simplistic view. Several labs have attempted to clone this single dominant gene, but none has been successful. The recent use of molecular markers to characterize segregating populations of apomictic and sexual individuals has revealed that apomixis often co-segregates with a large chromosomal segment (~25-40 cM in *Pennisetum squamulatum*) which shows a strong suppression of recombination (Ozias-Akins et al. 1998). It is possible that many genes are contained in this one unit, all of which would co-segregate as a single factor, i.e. a haplotype or “linkage block.” Work in many

diverse species supports this finding, including apospory in *Tripsacum dactyloides*, *Paspalum simplex*, and *Brachiaria decumbens*, and diplospory in *Erigeron annus* (Grimanelli et al. 1998a, Pupilli et al. 2001, Pessino et al. 1998, Noyes and Rieseberg 2000). It has not been determined whether this suppression of recombination is necessary for apomictic function or whether it is an evolutionary by-product of long-term asexual reproduction (Judson and Normark 1996). Additionally, researchers in the Netherlands have managed to genetically "split" apomeiosis and egg parthenogenesis in at least one apomictic species. In one study diplosporous *Taraxacum* plants were crossed to their sexual relatives and the progeny scored for sexuality over several generations. In the third generation researchers discovered plants that were diplosporous but lacked the parental parthenogenic capabilities. These results imply that, at least in this species, apomixis is determined by more than one gene (van Dijk et al. 1999).

As briefly mentioned earlier, most apomicts are polyploid, and this can often obfuscate any genetic analyses. Though this relationship is well documented, there is some debate as to the exact causal relationship, if in fact any, between the two conditions. Some apomictic species are diploid, e.g. *Arabis holboellii*, *Potentilla argentea*, and *Hierochloe australis* (Sharbel and Mitchell-Olds 2001, Asker and Jerling 1992), so polyploidy is not required for apomictic reproduction. In addition, if apomixis were initially present in a diploid species then it can be assumed, due to the presence of facultative sexuality, fertilization of unreduced egg cells with reduced or unreduced pollen would generate polyploids at a higher frequency than that commonly seen in sexual species. As polyploids are typically more competitive than diploids, it is

conceivable that polyploids would eventually become the dominant presence in a ploidy-diverse apomictic population. This would also explain why some apomictic species display a variety of ploidies—the incidental fertilization of unreduced egg cells could happen multiple times and generate ever-increasing ploidies (Bicknell and Koltunow 2004). This theory is supported by recent work in *Hypericum* that showed a correlation between genome size and evolutionary age in several different apomictic groups (Matzk et al. 2003).

In contrast, many researchers have hypothesized various ways a step-wise evolution of apomixis could result in co-evolution of polyploidy. In one sample scenario an individual plant would first become capable of parthenogenesis (meaning it would initially produce less competitive haploid seed). This plant or its progeny would then acquire the ability to produce unreduced egg cells. Initially these plants would produce triploid offspring, and if the ability to produce unreduced egg cells was retained across generations, the ploidy would increase with each generation. Finally, fertilization of the egg cell would be inhibited by some mechanism and the current level of polyploidy would be locked in place. This can occur quickly or over the course of several generations. Other scenarios are possible, but in most of these formulations it is almost required that polyploidy plants would be generated in one or more of the more intermediate steps (Barlow 1959, Powers 1945).

There is also significance evidence that apomixis can result directly from polyploidization. Polyploidization can affect global gene expression levels through a varity of mechansism, including dene deletion, mutation, or multiple forms of epigenetic

modification (Adams et al. 2003). Any or all of these changes could conceivably deregulate sexual reproduction enough to allow apomictic reproduction to occur (Carman 2001). For example, pairing in a newly formed allopolyploid could be disrupted to the point where unreduced gametes are produced at high frequency, opening the door for other parts of apomictic reproduction. It has been demonstrated in at least one instance that autopolyploidization of a diploid sexual species results in apomictic tetraploids (Quarin and Espinoza 2001). This becomes even more feasible when one realizes that allopolyploidizaion would result in even more genetic mismatch, mis- or de-regulation, and *de novo* reproductive events.

In the wake of such contradictory findings it is becoming more and more evident that the regulation of apomictic vs. sexual reproduction may be more complicated than anyone first realized. It is entirely possible, indeed, probable, that the mechanisms of genetic regulation and control are as numerous and as varied as the physiological mechanisms outlined in the beginning of this section. Further research is needed to sort out the contradictory information and determine the genetic source(s) of apomixis. Only when this picture is complete will we be able to use apomictic reproduction to improve agricultural production.

Efforts to Introduce Apomixis into Agriculturally Important Species

Agriculture involves domestication and improvement of wild plant species. Thousands of years of human selective pressure have yielded modern crops that feed an ever-growing population. Apomixis could prove to be very beneficial to modern

agriculture in several ways, e.g. stabilizing heterozygous populations over multiple generations, rapid production of fixed genotypes from segregating populations, reproductive isolation (maternal and paternal) of genetically modified organisms, etc. Perhaps one of the most prominent uses would be the trans-generation fixation of hybrid vigor. The concept of hybrid vigor was first quantified by in 1908 by George H. Shull, who found that that when two inbred lines were crossed, the resultant hybrid progeny were of increased size relative to both parent varieties (Poehlman 1959). However, the current processes used to produce hybrid maize are rigorous and expensive. Additionally, the hybrids are unstable; their seed cannot be kept for planting the next year because the progeny segregate for the inbred line traits and are less desirable than the parental hybrids. Therefore, the farmer must buy seeds year after year. Likewise, the seed company must generate huge quantities of hybrid seeds through an arduous process of selection and pollination (Koltunow 1995). If apomixis were transferred or recreated in crops that demonstrate hybrid vigor, the heterozygosity of the hybrid population would be "fixed," i.e. the traits would not segregate in the asexually produced generations.

Apomictic reproduction would also be beneficial to agricultural varieties produced using molecular cloning and transformation. These transgenic crop species are problematic for two reasons. As with hybrids, these crops suffer from sexual segregation of introduced traits. For example, transgenic maize can cross-pollinate with other varieties to produce less vigorous progeny, again requiring the farmer to purchase fresh seeds year after year. Secondly, there is a growing concern in the world that genes

introduced into crop species can "escape" into wild-type species (Ellstrand 2001). However, if these varieties were engineered to produce asexually they could be reproductively isolated, thereby eliminating the contamination in both areas. True parthenogenic apomicts could be engineered to be male sterile, which would reduce the probability of trangenes “escaping” into wild populations. Pseudoparthenogenic varieties could also be male-sterile and planted interspersed with their non-transgenic, male-fertile relatives. These plants would pollinate the transgenics and initiate embryo development, but the progeny would remain genetically unaltered. Unfortunately, the above scenarios are based on theory and speculation. They have yet to be proven experimentally because no sexual crop species has been successfully converted to asexual reproduction, despite several decades of effort.

Initial attempts focused on breeding apomixis into sexual species from the closest apomictic relative (e.g. transferring apomixis from *Tripsacum dactyloides* to *Zea mays* (Dewald and Kindiger 1994) or from *Pennisetum squamulatum* to *Pennisetum glaucum* (Dujardin and Hanna 1989)). However, these attempts have met with little success. Such projects are often plagued with low seed viability and limited reproduction, sexual or asexual, in individual plants (Spillane 2001). Additionally, efforts to cross *Tripsacum* to maize have reached an impasse where the plants apparently must contain at least eight different, specific chromosomes in order to express apomixis (Lukina and Chistyakova 1999). Such a high number of chromosomes means these plants contain large numbers of undesirable characteristics from the uncultivated parent. Many researchers have been

forced to look for alternatives to traditional breeding strategies for the successful introduction of apomixis into sexual plant species.

More recently, many labs have focused their efforts on cloning the gene(s) responsible for apomictic reproduction in the hopes they can be effectively transformed into sexual species. The idea seems most feasible in those apomicts where the trait segregates as one unit linked to a small number of markers, e.g. *Pennisetum squamulatum*, *Tripsacum dactyloides,* and *Taraxacum officinale*, among others (Bicknell and Koltunow 2004). However, FISH and physical mapping efforts have demonstrated that although these regions may be linked to a small number of markers, they often span many megabases of DNA with low rates of recombination (Ozias-Akins et al., 1993, Roche et al., 2002, Grimanelli et al., 1998b, Blakey et al., 2001, van Dijk et al., 2003). Though this does not completely eliminate the possibility of cloning the genes from these regions, it does make the project more difficult. Not only are more cloning and sequencing required, but one can also presume many genes and pseudogenes are present in this region. If so, identifying the relevant genes and determining their function may be more difficult. In addition, the difficulty in transforming apomixis genes into sexual species increases proportional to the number of genes, as do the problems of coordinating the expression patterns and avoiding gene silencing (Dresselhaus et al., 2001). Admittedly, at the current time these problems are mostly pessimistic speculation, and if they do arise they will be addressed. Even if it proves unfeasible to use gene transformation to convert sexual species to asexuality, the study and sequencing of the

apomixis-linked genetic regions from these species will provide a wealth of important information.

Alternatively, it may be that engineering the switch from sexual to asexual reproduction will not involve the use of apomictic species at all. There are several proposed techniques that are being used in attempts generate *de novo* apomixis in sexual species. The earliest attempts at accomplishing this involved screening mutagenized populations of *Arabidopsis thaliana* for parthenogenic seed production (i.e. screen a mutagenized male sterile line for fertility). It was initially hoped that through this method one would discover apomictic *Arabidopsis* mutants from which researchers could clone the genes involved in its synthesis. Though this method has yielded several genes of interest, specifically the *FIS, FIE,* and *MEDEA* genes that regulate early endosperm development, it has not uncovered any genes relating to retention of diploid chromosome number or inhibition of egg fertilization (Ohad et al., 1996, Chaudhury et al., 1997). The inverse of the above mutagenesis screens has also been attempted in a few apomictic species. Grimanelli et al. (2001) used *Mutator* transposable elements to mutagenize a population of asexual *Tripsacum*/maize hybrids, then screened for loss of asexuality, while Bicknell et al. (2001) did roughly the same thing with *Ac/Ds*-like elements in *Hieracium*. While these screens have resulted in the identification of interesting mutations, no completely sexual mutants have been identified. In addition, characterization and cloning of the genes responsible has progressed very slowly (Weld et al., 2002), due to genetic complexity of these species and their lack of in-depth genetic characterization.

Finally, some researchers have found limited success using reverse genetics approaches to generate apomixis in the model species *Arabidopsis thaliana*. Using a cDNA-AFLP method to characterize genome wide expression levels, Albertini et al. (2004) isolated several ESTs that were differentially expressed between apomictic and sexual individuals in a segregating population of *Poa pratensis*. These ESTs were used to identify two genes, *PpSERK* (*Somatic Embryogenesis Receptor-Like Kinase*) and *APOSTART*, which seem to initiate and regulate apomictic reproduction (Albertini 2005). When the *Arabidopsis* homologue of *PpSERK* (*AtSERK1*) was expressed under the control of an early ovule development specific promoter there was some evidence of apomictic reproduction in the F2 generation, but this did not persist in the F3 progeny (Kantama et al., 2006). Research into the functions of these genes and their other homologs is continuing. In addition, it has also been postulated that known and characterized mutations from kingdoms outside of Plantae can be related to the genetic mechanisms that produce apomictic reproduction. It is thought that homologous genes could be identified in *Arabidopsis* and their role in plant reproduction elucidated and manipulated (Vielle-Calzada 1996). Currently, none of these techniques has successfully converted a sexual species to asexuality, either in an agronomic crop or in a model organism. However, each has contributed significantly to our understanding of the problem and it may be that, in the end, only a combination of all of them will succeed.

Semigamy Mutant of Cotton

The study of reproductive mutants can yield valuable information about sexual reproduction in general and also aid in determining what changes are necessary to successfully produce asexual offspring. There are several documented mutations in various plant species that mimic various parts of apomictic reproduction, including *failure of cytokinesis* (*fc*) and *omission of second meiotic division* (*os*) in potato (lead to formation of 2n megagametophytes and thus 2n egg cells), *ameiotic-1* (*am1*), *ameiotic-2* (*am2*), and *elongate* (*el*) in maize (also yield 2n egg cells), and *haploidy initiator* (*hap*) in barley (parthenogenic) (Peloquin et al., 1999, Curtis and Doyle, 1991, Hagberg and Hagberg, 1981, Rhodes and Dempsey 1966). In addition, the *Semigamy* (*Se*) mutation from cotton is a naturally occurring mutation that duplicates a method of genetic isolation seen in many apomictic species.

Semigamy was first characterized in various recurrently apomictic species of *Rudbeckia* (Battaglia 1946) and more recently in *Cooperia pendunculata* (Coe 1953) and *Zephyranthes microsiphon* (Crane 1978). Semigamy has also been induced by gamma radiation in *Arabidopsis thaliana* (Gerlauch-Crus 1970). In these apomictic species, semigamy results in the sperm nucleus being sequestered into a developmentally inactive portion of the embryo. Paternally derived haploid cells may divide an indeterminate number of times, but they do not form any part of the viable embryo. Instead, they are isolated near to or as part of the suspensor, a small organ that interconnects the embryo and the endosperm (Coe 1953). Two naturally occurring semigamous mutants that that yield parentally chimeric progeny have been reported in naturally occurring sexual

species: the *Semigamy* mutant of *Gossypium barbadense* (Turcotte and Feaster 1963), and a mutant of *Theobroma cacao* (Lanaud 1988).

The *Semigamy* (*Se*) mutant of cotton differs from these natural forms in that the paternal genome is often a part of succeeding generations. When initially recovered in doubled-haploid lines from *Gossypium barbadense* commercial variety Pima S-1 (Turcotte and Feaster 1963), *Se* mutants were found to produce progeny with large numbers of haploid plants that could be descended from either the maternal or the paternal parent. In addition, mutant lines also produced large numbers of chimeric offspring. The sectors in these plants were a mix of maternal haploid, paternal haploid, and tetraploid genomes. However, only the tetraploid plants or sectors were fertile, and these areas had obviously undergone some form of non-apomictic genomic mixing. The only true apomictic offspring produced by the *Se* mutant were sterile, making this a non-recurrent form of apomixis.

Initial characterization of homozygous *Se* mutant lines indicated that they produced from 32 to 61% haploid progeny (Turcotte and Feaster 1963). This wide variance in levels of trait expression has been documented in other studies, and implies the existence of several environmental or genetic factors that may influence *Se* expression (Turcotte and Feaster 1963, Turcotte and Feaster 1974, Zhang et al. 1999, Zhang and Stewart 2004). However, crosses between homozygous *Se*, heterozygous, and wild-type plants have indicated that the *Se* phenotype is conditioned by one incompletely expressed dominant gene. Several data support this hypothesis. The levels of haploid production were significantly higher in *SeSe* x *SeSe* crosses than in *SeSe* x

sese crosses, but the latter did produce haploid and chimeric offspring. Interestingly, *sese* x *SeSe* crosses produced no haploid progeny, indicating that the *Se* allele must be transmitted through the female gamete in order to be expressed. However, having mutant *Se* alleles present in both gametes increases the frequency of expression. When selfed, the heterozygous F1 progeny from *SeSe* x *sese* crosses produced an F2 population that segregated 3 : 1 haploid producing : non-haploid producing plants, and when the F1 plants were backcrossed to the non-semigamous line only 50% of the progeny were capable of haploid production (Turcotte and Feaster 1974, Chaudhari 1978, Zhang et al. 1999).

However, there are other ways to explain the above observations, especially when one considers all the possible genomes involved in sexual reproduction in plants—male sporophytic (2n), female sporophytic (2n), male gametophytic (n), female gametophytic (n), and zygotic (2n). The above results would also support the argument that the *Se* gene is expressed in the gametophytes, where it is in a hemizygous state (thus completely undermining the question of dominant or recessive mode of action). Alternatively, *Se* expression could be determined in the zygote with some residual maternal and paternal effects. More research is needed to refine our understanding of the genetic regulation of the *Se* gene.

All studies to date on *Se* expression have relied on genetic data and progeny evaluation. Newly developed techniques of cytological evaluation have revealed much about how *Semigamy* functions in the cotton mutants. It is our belief that, if applied, cytological evaluation could also help to explain the precise mode of action of the

mutant *Se* allele. In addition, cytological evaluation may provide a useful screening technique to better qualify individual genotypes in populations segregating for semigamous reproduction.

Despite the lack of information about genetic regulation and mode of action of the *Semigamy* gene, it has been put to a variety of uses by cotton breeders and researchers. *Semigamy* has been used to produce doubled-haploid lines in both *G. barbadense* and *G. hirsutum* that show great potential in breeding for fiber qualities (Mahill et al. 1984). It has also been used in the construction of a *G. barbadense* x *G. hirsutum* genetic linkage map (Zhang et al. 2002). Cotton physiologists have also used the chimeras produced by semigamous lines to study relative levels of cellular interactions between different developmentally derived layers of mature plants (Dolan and Poethig 1998). Lastly, there are projects under development that aim to use semigamous lines for the efficient mass production of doubled haploid lines (Stelly et al. 1988). However, we believe that the potential uses of the *Se* mutant can be significantly increased through a more thorough understanding of the genetic mechanisms regulating its expression.

A CYTOLOGICAL EVALUATION OF THE *SEMIGAMY* MUTATION OF PIMA COTTON (*GOSSYPIUM BARBADENSE*)

Introduction

Deviations from normal reproductive pathways can be used for a variety of purposes by researchers, breeders, and farmers. These mutants allow researchers to dissect plant reproduction into its respective components for easier analysis. Additionally, some mutations generate useful phenotypes that can be used by breeders and farmers to quickly and easily produce valuable offspring. However, efficient exploitation of such mutants depends on a thorough understanding of their function and mode of action. Unfortunately, the determination of these factors is complicated and problematic, as many of the events surrounding plant reproduction, e.g. meiosis, sporogenesis, gametogenesis, fertilization, zygote development, etc., occur in a small number of cells only at very specific, short-lived time points. Special techniques must be devised to discover and characterize these potentially beneficial reproductive mutations. Ideally, a better understanding of the processes behind plant reproduction would help enable scientists to "customize" plants to produce the optimal types and numbers of given progenies that would be most useful to breeders and producers.

One mutant with much potential for the cotton industry is the *Semigamy* mutation (*Se*). When discovered in 1963 from the commercial Pima S-1 variety of Pima cotton (*Gossypium barbadense*), *Se* mutants were initially characterized as producing large numbers of haploid offspring (Turcotte and Feaster 1963). Further analyses revealed that the plants also produce chimeric offspring with a mix of maternal haploid, paternal

haploid, and/or zygotic sectors (Turcotte and Feaster 1967). All of these offspring proceeded from monoembryonic seed. Such a phenotype is consistent with the naturally occurring semigamous mode of reproduction documented in *Rudbeckia* (Battaglia 1945), *Cooperia* (Coe 1953), and *Zephyranthes* (Crane 1978). In these species a pollen cell fuses with the egg cell (as in normal fertilization) but the resulting heterokaryon forgoes normal karyogamy, or the natural fusion of egg and pollen nuclei. Instead, the two nuclei undergo separate simultaneous mitotic divisions resulting in four haploid cells. The paternally descended cells are developmentally isolated while the maternal cells grow and divide to produce an embryo that is an exact clone of the maternal plant (Battaglia 1945). This is a pseudogamous form of apomictic reproduction, or asexual reproduction through seed (Nogler 1984), because fertilization is required for the transition from egg cell to zygote. However, the effective sequestering of the paternal genetic material ensures that all progeny produced are genetically identical to the maternal parent.

The high numbers of progeny with maternal, paternal, and zygotic sectors indicate that the *Semigamy* mutation acts through a developmental pathway similar to the one outlined above (Turcotte and Feaster, 1967; Chaudhari, 1978). However, the presence of zygotic and paternal haploid sectors and plants indicate that the paternal genome segregation is not completely effective. Turcotte and Feaster (1973) hypothesized that the chimeric plants could have been created through some ill-defined mechanism involving polyspermy, but recent cytological evidence has conclusively demonstrated that this is not the case. Instead, the semigametic cotton egg cell is

fertilized by one pollen nucleus, which, as in other semigamous species, remains adjacent to but separate from the egg cell nucleus (Figure 1A). Approximately 95 to 125 hours after anthesis, the sperm and egg nuclei undergo synchronized mitotic divisions. Each nucleus forms its own spindle and mitotic plate. However, this initial mitotic division could result in one of three separate outcomes. Most of the egg cells divide to form four haploid daughter cells—two maternal in origin and two paternal (Figure 1B). In a significant portion of egg cells, the microtubule-organizing centers are aligned close enough together that at telophase one nuclear membrane forms at each pole. This results in two haploid chromosome sets—one maternal and one paternal—fusing into one tetraploid zygotic nucleus at each pole. The egg cell then divides in half instead of quarters, yielding a two-celled tetraploid embryo (Figure 1C). Lastly, in rare cases the separate mitotic spindles of the egg and pollen nuclei align in such a way that the tetraploid zygotic nucleus can only form at one spindle pole. This results in the formation of a three-celled embryo with one tetraploid zygotic cell, one maternally derived haploid cell, and one paternally derived haploid cell (Figure 1D). The first and last of these possible outcomes can produce a wide variety of different chimeral combinations depending on which type(s) of cells compose the apical meristem (Hodnett 2006). The net result from this is a form of non-recurrent apomixis. The truly apomictic plants or sectors, i.e. those derived solely from the maternal plant, are haploid and sterile while the fertile, tetraploid plants or sectors are not asexually derived and thus contain genetic material from both parents.

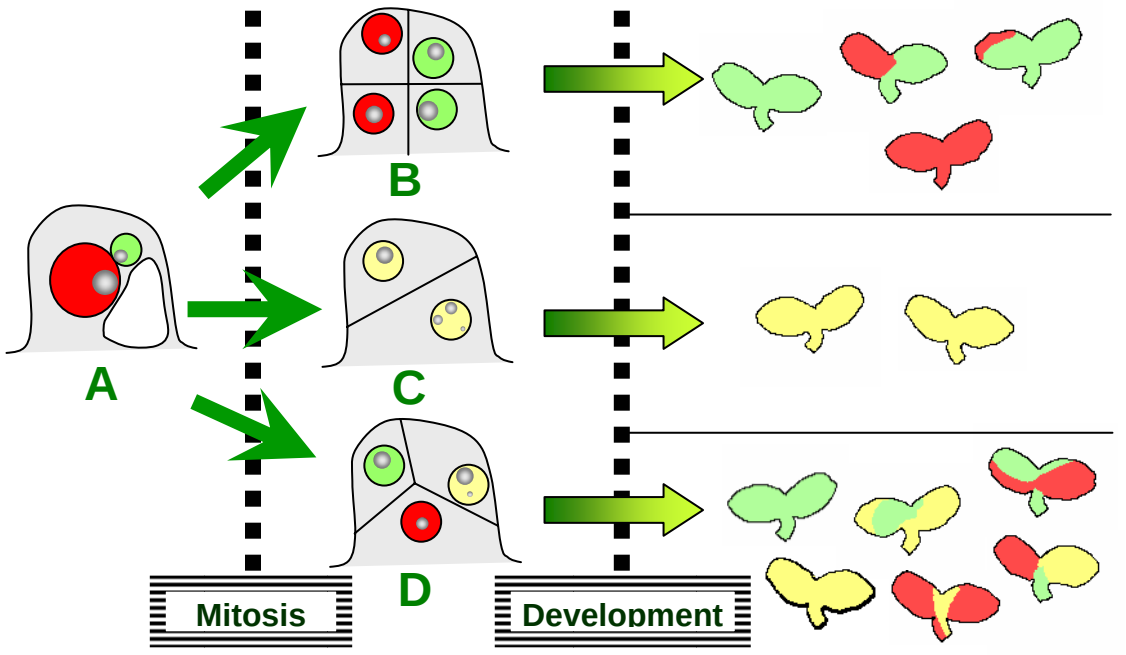


Figure 1. Schematic Representation of Possible Chimeras Arising After Semigametic Reproduction.

(A) Original semigamous egg cell. In the second column are the possible results of the first mitotic division: (B) chimera with two maternal haploid and two paternal haploid cells, (C) zygote with two tetraploid cells, and (D) tri-chimera with one maternal haploid, one paternal haploid, and one zygotic diploid cell. Rightmost are subsets containing possible haploid, chimeric, and trichimeric seedlings that might arise as a result of embryo development.

To date, the timing and mode of action of the mutant allele of the *Se* gene has been based solely on progeny scoring. The frequency of haploid production remains relatively constant in successive generations of the original doubled haploid line 57-4 (derived from the commercial variety Pima S-1), indicating that this line is homozygous for the haploid producing mutation (Turcotte and Feaster 1963). Crosses with this line

resulted in high haploid production if the male was homozygous for *Semigamy* and intermediate levels if a wild-type male was used. Though the gene can be transmitted thorough both parents, *Semigamy* is not expressed if it is not present in the female parent, i.e. wild-type females produced little or no haploid offspring when crossed to heterozygote or homozygous mutant males. Progeny from selfed heterozygote plants (F2 plants from a 57-4 x Pima S-1 cross) segregated 3:1 for semigamous (haploid producing) and non-semigamous lines, while progeny of heterozygotes crossed to homozygous mutants segregated at 1:1 (Turcotte and Feaster 1974, Zhang et al. 1999). Additionally, heterozygous plants can produce haploid progeny when used as females in crosses with heterozygous or homozygous mutant males, although the frequencies of haploid production are overall lower than the frequencies seen when a homozygous female is used. These data indicate that *Semigamy* is controlled by one incompletely dominant gene (Zhang and Stewart 2004).

However, measuring haploid production as a means of detecting semigamy has several drawbacks. Though true-breeding semigamous lines produce haploids and chimeras in every generation, the frequency of their production can vary widely. In the first reports on the *Semigamy* mutation, the levels of haploid production in homozygous mutants varied from 25% to 60% (Turcotte and Feaster 1963). More recent studies have indicated that these differences are due not only to environmental effects, but that significant differences exist between frequency of haploids produced by different plants in the same fields and even between different bolls on the same plant (Zhang and Stewart 2004). These results indicate there are strong developmental and/or

environmental effects that may be affecting this phenotype. Additionally, progeny scoring can be affected by lower haploid germination rates, the possibility of zygotic sectors out-competing neighboring haploid sectors in the apical meristem so that the chimeric phenotype is not immediately noticeable, and the difficulty in consistently identifying haploids at the seedling stage.

In addition, scoring only the plants produced by certain genotypes fails to effectively differentiate the plants that express a gene from those that that merely carry it. Any heterozygote produces plants that express a certain phenotype—the question of dominance or recessiveness is determined by whether or not the heterozygote itself expresses the trait. In most cases this can be determined by looking at the ratios of mutant vs. wild-type progeny (1:3 for a recessive trait, 3:1 for a dominant trait), but given the previously discussed instability of *Semigamy* expression, the ratios rarely fit the standard, easily explained patterns. As a result, the relatively straightforward question of determining the mode of *Semigamy* expression has been a subject of much debate over the last four decades.

To better characterize the *Semigamy* mutation of Pima cotton our lab devised a method that enables us to directly examine the egg cell before, during, and after fertilization (Hodnett et al. 1997). This allows us to directly observe the *Semigamy* phenotype as it occurs. We used this procedure to analyze a large number of ovules from various intercosses between homozygous mutants, heterozygotes, and wild-type plants in a concerted effort to better determine the timing and mode of action of the mutant *Semigamy* allele as compared to the wild type. It is our hope that identifying the

mode of action of the *Semigamy* gene will contribute to the usefulness of this interesting mutation in various cotton breeding and improvement programs as well as advance our overall knowledge of the pathways involved in plant reproduction.

Materials and Methods

Plant Growth Conditions

Homozygous semigamous seed stocks were kindly provided by Dr. J. McD. Stewart at the University of Arkansas, Fayetteville, AR (57-4, genotype $r_1r_1SeSeV_7V_7$) and Dr. Liam Dolan, then at Pennsylvania State University, University Park, PA (Sev_7 marker line, genotype $r_1r_1SeSev_7v_7$). The *Semigamy virescent-7* marker line contains a recessive chlorophyll mutation that gives the plants a light green coloring. It was first created by Turcotte and Feaster (1973) and used to identify the origins of haploid and zygotic sectors in chimeric plants. The heterozygous lines used in this study ($R_1r_1SeseV_7V_7$) were the product of second-generation backcrosses (BC2F1) between a red plant semigamous marker line (derived by Dr. David Stelly, genotype $R_1R_1SeSeV_7V_7$) and Pima S-6. The wild-type plants used in this study were from varieties Pima S-6 and Pima S-1 (both genotype $r_1r_1seseV_7V_7$). All lines were planted in Jiffy pellets, then transferred into pots and grown in our winter greenhouses in College Station, TX.

Sample Collection and Processing

Upon maturity the plants were crossed in a standard 3x3 factorial mating scheme (Table 1). The resulting F1 crosses were all harvested ~72 hour after fertilization. The ovules were excised from the ovaries and processed as described elsewhere (Hodnett 1997, Hodnett 2006). Briefly, the ovules were fixed for at least 24 hours in a 10x

volume of FAA_{50} (10% formalin, 5% glacial acetic acid, 50% ethanol (95%), 35% H_2O) before the integuments were removed with the aid of a dissecting microscope. The nucelli were hydrated in a series of 50% EtOH, 25%, EtOH, and pure H_2O (30 minutes per solution) and stained overnight in 0.08% azure C in 0.05M phosphate buffer (pH 7.0). The following day the nucelli were dehydrated in a series of ethanol washes (from 10% to 95% EtOH in 10% increments) for 15 minutes each. Destaining also occurred during these washes. Acetic acid (1%) was added to every fourth wash if destaining was not occurring fast enough. After a final wash in 95% EtOH, the nucelli were washed successively in 5:1, 2:1, 1:1, 1:2, 1:5, 0:1, and 0:1 EtOH (95%) to methyl salicylate (22 minutes for each step). The nuclei were allowed to clear overnight then examined under a Zeiss Universal II microscope using either a 63x Neofluar objective (N.A. = 1.25, W. D. = 0.5 mm) kindly provided by Dr. Spencer Johnston, Texas A&M University, College Station, TX. Images were obtained through an Optronics VI-470 CCD camera (470 line, RGB/grayscale) attached to a 0.7-7 x zoom lens system. Digital images were produced by averaging eight still shots and were adjusted for brightness and contrast using both camera controls and, in some cases, Adobe Photoshop™ software.

Statistical Analysis

All ovules from one ovary were stained and scored together. This study was conducted in a double-blind manner; the vials were assigned random numbers by a disinterested third party so that the researcher was unaware which type of cross was represented in each. Fertilization and/or the *Semigamy* phenotype were scored by eye

and the data recorded directly into a Microsoft Excel worksheet. Statistical calculations were performed using the standard Excel formula function.

Table 1. Number of Ovules Analyzed for Each Type of Cross in a 3x3 Factorial Mating Scheme Used to Analyze the Frequency of Semigamous Ovule Production

Female Genotype	Male Genotype		
	SeSe	*Sese*	*sese*
SeSe	453	322	184
Sese	315	141	369
sese	390	268	209

Results

Detection of the Semigamy Phenotype

As previously documented in the literature, our stain clearing technique allowed for efficient and reliable differentiation of unfertilized, sexual, and semigamous ovules (Hodnett 1997, Hodnett 2006). Ovules were considered fertilized if a pollen tube was present in the micropylar end and the cytoplasm of the egg cell was condensed with only a small micropylar vacuole. Ovules were scored as unfertilized if the egg cell was still large and balloon-like or if two synergids and two polar nuclei were immediately obvious (Figure 2A). Fertilized ovules were further classified as sexual or semigamous. Ovules were scored as semigamous only if the male nucleus was present and readily identifiable (Figure 2B). Sexual ovules were divided into two categories: definitely sexual—one large, oval nucleus with multiple nucleoli (Figure 2D)—or probably sexual, meaning the cell had one round nucleus with only one nucleolus and no sperm nucleus present (Figure 2C). For the purpose of analysis, the latter two categories were grouped together into one sexual grouping. Over 30 ovaries were harvested for each type of cross, resulting in over 100 fertilized ovules being scored for each (Table 1).

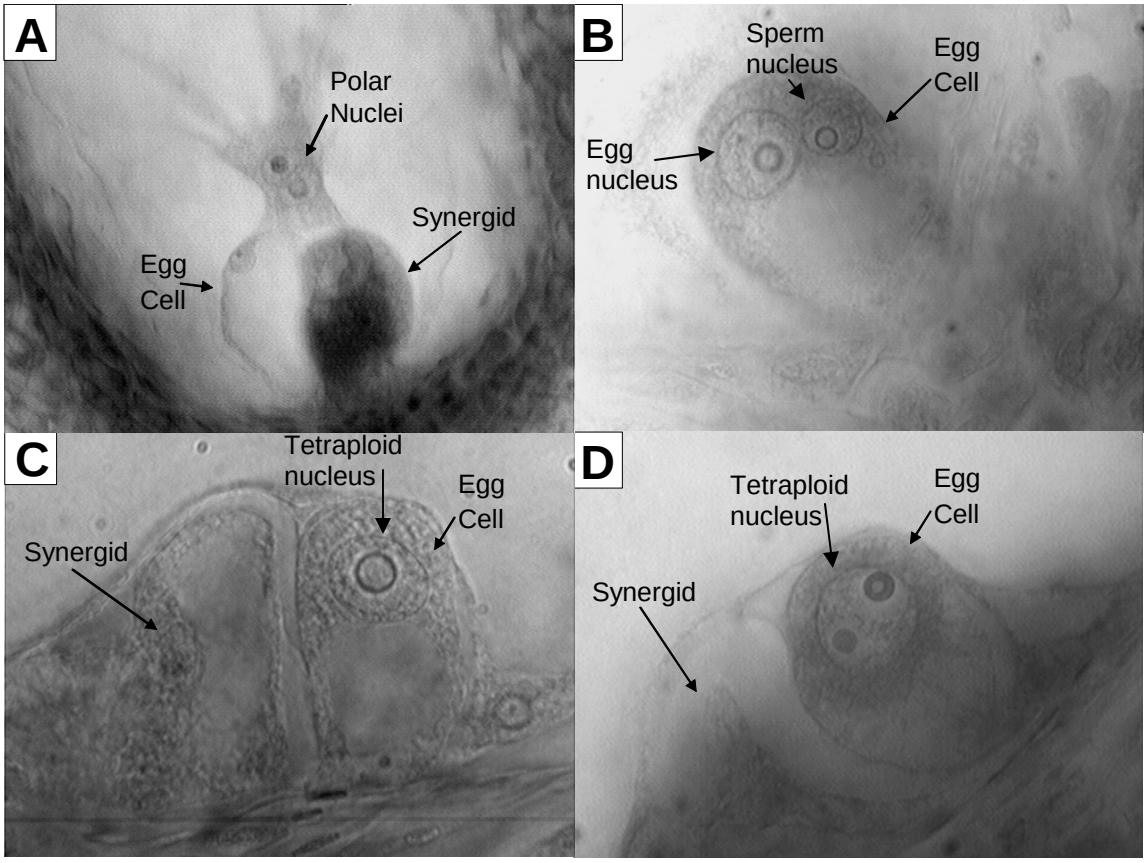


Figure 2. Representative Nucelli.

(A) An unfertilized nucellus. Notice the egg cell is mostly a large vacuole with only a thin layer of cytoplasm. The egg nucleus is small and spherical. The visible synergid is also large (second is out of the plane of focus) with a vacuole in the chalazal end. (B) A semigamous nucellus. In a fertilized egg cell, the cytoplasm condenses while the vacuole becomes much smaller and is relegated to the micropylar end of the egg cell. In a semigamous zygote both the egg and sperm nuclei are quite round. The egg nucleus is the larger of the two (on the left). Though the nuclei are adjacent to each other, no fusion has occurred. (C) A sexual zygote with one nucleolus. The zygotic nucleus is larger than an unfertilized egg nucleus and more oval shaped. There is only one remaining synergid, which is highly condensed. (D) A sexual zygote with two nucleoli. In this zygote the oval shape of the nucleus is even more pronounced. The presence of two nucleoli indicates that karyogamy has recently occurred.

Mode of Action of the Semigamy *Mutant Allele*

The overall results from our 3x3 factorial mating design are summarized in Table 2. The frequency of semigamous ovule production was calculated by dividing the numbers of semigamous ovules by the total number of fertilized ovules (semigamous + sexual, Table 1). The results from each type of cross are summarized briefly below.

Table 2. Frequencies of Semigamous Ovule Production by Various Crosses Between Homozygous Mutants, Heterozygotes and Wild-Type Plants

Female Genotype	Male Genotype		
	SeSe	*Sese*	*sese*
SeSe	0.94	0.45	0.10
Sese	0.43	0.23	0.04
sese	0.03	0.02	0.01

This study utilized two different wild-type lines: Pima S-6 and Pima S-1. There were no significant differences between these two lines when they were used in *sese* x *sese* crosses, or when they were used as males or females in crosses with heterozygote or mutant plants ($p > 0.1$ for all), so for the purposes of this study data from these two lines

were grouped together (Table 3). We also compared the frequencies of semigamous reproduction in three different types of crosses: non-emasculated flowers allowed to self-pollinate, emasculated flowers pollinated with an open flower from the same plant, and emasculated flowers pollinated with pollen from a different wild-type plant. The crossing method had no significant effect of *Semigamy* expression ($p > 0.1$, Table 4). Overall, the frequency of *Semigamy* expression in wild-type x wild-type crosses was 0.01 and was not significantly different from zero ($p > 0.1$).

Table 3. Comparison of *Semigamy* Frequencies in Pima S-1 (PS-1) and Pima S-6 (PS-6)

	PS-1 x PS-6	PS-1 x PS-1	Female = PS-1	PS-6 x PS-1	PS-6 x PS-6	Female = PS-6	Total
Number Ovules	23	64	87	15	274	289	376
Number Fertilized	18	44	62	14	133	147	209
Frequency of Fertilization	0.78	0.69	0.71	0.93	0.49	0.51	0.56
Number Semigamous	0	1	1	0	1	1	2
Frequency of Semigamy	0.00	0.02	0.02	0.00	0.01	0.01	**0.01**

Table 4. The Effects of Selfing and Crossing Methods on *Semigamy* Expression in Wild-Type Plants

	Selfed	Crossed to Self	Crossed to Another *sese*	Overall
Number Ovules	230	87	59	376
Number Fertilized	103	56	50	209
Frequency of Fertilization	0.45	0.64	0.85	0.56
Number Semigamous	1	1	0	2
Frequency of Semigamy	0.01	0.02	0.00	**0.01**

In crosses involving homozygous mutants, two different lines were also used: 57-4, the genetic standard line that is isogenic to Pima S-1, and Sev_7, a semigamous marker line homozygous for a recessive chlorophyll mutation. Our results show that 57-4 shows 0.96 frequency of semigamous ovule production when used as a female in homozygous mutant crosses (*SeSe* x *SeSe*), while Sev_7 exhibits 0.90 when used as female (Table 5). These proportions are significantly different ($p < 0.05$). At this time we are unsure why such a difference exists, as Sev_7 was derived from 57-4 (Turcotte and Feaster 1969). The largest deviation produced by the various crosses is seen when Sev_7 is used as female in a cross with 57-4 (frequency = 0.82). There was no difference when these lines were used as females or males in crosses with heterozygotes, or when they

were used as males in crosses with wild-type plants ($p > 0.1$ for all). However, there was significant difference between these two lines when they were used as females in crosses with wild-type plants (frequency of semigamy equals 0.21 when 57-4 is used (of 78 fertilized ovules), and 0.02 when Sev_7 was used (106 fertilized ovules)). Lastly, our analysis showed that the type of cross (selfs, intra-, or inter-crosses) had no effect of the levels of *Semigamy* expression ($p > 0.1$, Table 6).

Table 5. A Comparison of the Effects of Different Homozygous Mutant Lines on *Semigamy* Expression

	57-4 x 57-4	57-4 x Sev_7	Female = 57-4	Sev_7 x Sev_7	Sev_7 x 57-4	Female = Sev_7	Total
Number Ovules	447	107	554	228	63	291	845
Number Fertilized	202	63	265	149	39	188	453
Frequency of Fertilization	0.45	0.59	0.48	0.65	0.62	0.65	0.54
Number Semigamous	191	63	254	138	32	170	424
Frequency of Semigamy	0.95	1.00	0.96	0.93	0.82	0.90	**0.94**

Table 6. Effects of Fertilization Method on *Semigamy* Expression in *SeSe* Plants

SeSe x *SeSe*	Selfed	Crossed to Self	Crossed to Another *SeSe*	Overall
Number Ovules	559	52	234	845
Number Fertilized	269	34	150	453
Frequency of Fertilization	0.48	0.65	0.64	0.54
Number Semigamous	251	31	142	424
Frequency of Semigamy	0.93	0.91	0.95	0.94

Overall, when homozygous lines were crossed to each other the frequency of semigamous ovule production was 0.96. This is much higher than the levels of haploids or chimeras produced by this cross that have been reported previously (Turcotte and Feaster 1963, Turcotte and Feaster 1974, Zhang 1999, Zhang 2004). This level is also significantly different from the frequency of 1.0 one would expect from a completely expressed mutation ($p < 0.01$).

When homozygous mutant plants were crossed with heterozygotes the frequency of haploid production dropped to half that seen in the *SeSe* x *SeSe* crosses—0.45 if a *SeSe* plant was used as female, 0.43 if it was used as male. There is no significant difference between these two frequencies ($p > 0.1$). However, there is statistical significance between both of these frequencies and 0.5 ($0.05 < p < 0.1$). When the

heterozygous plants were selfed or crossed to each other, the level of *Semigamy* expression was 23% and statistically insignificant from the expected 25% ($p > 0.1$). Lastly, when heterozygotes were used as females in crosses with wild type males (*Sese* x *sese*) 4% of the ovules were semigamous, a level significantly different from zero ($p > 0.1$).

If the female parent did not carry the mutant *Semigamy* allele, the levels of *Semigamy* expression were markedly low but did show some variation. If the male used was a homozygous mutant (*sese* x *SeSe*) the frequency of semigamous ovule production was 0.03, which was significantly different from 0 ($p < 0.01$). If a heterozygous male were used (*sese* x *Sese*) the frequency was 0.02 and was not significantly different from 0 or the level seen in crosses with a wild-type male (*sese* x *sese*, frequency = 0.01, $p > 0.1$).

Discussion

The pattern of *Semigamy* expression evidenced in our study most resembles two possible models: 1) a recessive gene that acts zygotically, so one copy must be inherited from each parent, or 2) gametophytically controlled expression of the *Semigamy* gene in both male and female gametes. A chi-squared analysis of these models failed to eliminate either hypothesis ($p < 0.01$). In the first model the mutant *Semigamy* allele is decisively recessive to the wild-type allele. If both are present in the zygote, only the wild type is expressed. In the second model the question of dominance remains unresolved as cotton gametes are hemizygotic—only one copy of each gene is present. If a diploid or disomic aneuploid gamete were to carry both a mutant and wild-type

allele, one cannot conclusively say which one would be expressed or what the overall result would be if such a gamete were to interact with a wild-type or mutant analog. Experiments have been proposed to test this scenario (Gwyn 1995), but to date none have been carried out due to difficulties inherent to doubling the cotton genome and the lack of a precise localization of the *Semigamy* locus. Yet it seems from our data that in most instances the presence of a wild-type allele of the *Semigamy* gene in the zygote, whether it is inherited from either parent, inhibits the expression of the mutant phenotype, i.e. the mutation is recessive to the wild type.

There exists a glaring contradiction to this conclusion—the high frequency (0.10) of semigamous ovules in a *SeSe* x *sese* cross. This result is also well supported in the literature—semigamous plants produce haploid and chimeric offspring when crossed as female with other non-semigamous lines (Turcotte and Feaster 1974, Mahill et al. 1984, Zhang et al. 2004). From these data it seems there is a strong maternal effect that serves to override the influence of the wild-type alleles inherited from the paternal parent. However, there is no evidence of a maternal effect when the homozygous female is used in crosses with heterozygous males; the frequency of semigamous ovule formation does not rise above 0.5. Additionally, any maternal effect present appears to also act recessively, as heterozygous females exhibit a substantial drop in semigamous ovule production when crossed with wild-type males (*Sese* x *sese*, frequency = 0.04), and there is no significant difference in the levels of semigamous ovule formation when heterozygous males or females are crossed with homozygous mutants (*Sese* x *SeSe* 0.43; *SeSe* x *Sese* 0.45). If the maternal effect were still present in the heterozygote plants one

would expect them to produce a higher percentage of semigamous ovules when they were used as females than when they were used as males, but this was not the case.

The means through which the maternal genotype somehow overrides the presence of a wild-type allele in the zygotic offspring has yet to be explained. This could perhaps be due to expression of the maternal *Semigamy* gene at some point during or after megagametogenesis or during fertilization. Additionally, studies in various model organisms show that maternally expressed genes can be loaded into developing egg cells regardless of their inherited genotype and expressed during zygotic development (Ray et al. 1996, Columbo et al. 1997). Therefore, the maternal effect evidenced by the *Semigamy* mutation may be due to the import of *Se* mRNA into the egg cell from the surrounding maternal tissue. This together with the *Se* transcripts produced by the gamete itself may override the genotype of the sperm nucleus. However, in the heterozygote the maternal mRNA would a mix of both *Se* and *se* forms, and perhaps the resulting levels of mutant expression would not be high enough to overcome the dominant *se* allele expressed by the male gamete. Alternatively, physical alterations of the organelles or cytoplasm of the egg cell could result from *Se* mutant expression in the megasporocyte. These changes could be established before fertilization and would presumably be uninfluenced by the genotype of the pollinating sperm nuclei. For example, alterations in the form of the cytoskeleton or the makeup of the mictrotubule organizing center (MTOC; the plant homolog of centrosomes) could impact nuclei motility at karyogamy. Likewise, changes in the MTOC can affect how nuclei behave at

the first mitotic division. More studies need to be done on the timing of *Semigamy* expression before any of these suppositions can be ruled out.

Most previous studies involving *Semigamy* have concluded that the mutant allele is incompletely dominant to the wild-type allele (Turcotte and Feaster 1974, Zhang 1999, Zhang 2004). Our data stand in near direct opposition to this finding. The difference comes from looking at two different phenotypes produced by the one mutant. Previous studies have relied on scoring the frequency of haploid production in lines carrying the *Se* mutation. Lines were scored as "semigamous" if they produced haploid or chimeric progeny and "sexual" if they did not. However, the classic definitions of the semigamy phenotype—the absence of karyogamy—is expressed during the zygotic stage of development, not the reproductive stage. These plants should not be scored on the basis of the progeny the produce but on the nature of their own zygotic history. In our study only those plant homozygous for the *Se* mutation displayed the *Semigamy* phenotype (discounting the as yet unexplained maternal effect). Heterozygous ovules were sexual. However, in previous studies heterozygous plants have been scored as semigamous because they expressed the "haploid-producing" phenotype. The phenotype was the result of one fourth of the plants they produced being homozygous mutants and therefore having the opportunity to be haploid or chimeric (depending on the outcome of the first mitotic division). However, our results show that these heterozygous plants themselves most likely never expressed the true semigamy phenotype as defined by Battaglia (1945), that is, the absence of nuclear fusion from the time of fertilization to the first mitotic

division. Thus, the *Semigamy* mutation should correctly be classified as recessive because its phenotype is not expressed in heterozygous plants.

By focusing our efforts on this specific cytological phenotype, we were able to remove many of the environmental and developmental effects that have clouded previous characterization efforts. Most notably, we have eliminated the factors affecting the outcome of the first mitotic division in the zygote. In a significant percentage of semigamous ovules this division can result in two diploid cells, an outcome which completely obscured the *Semigamy* phenotype (Hodnett 2006). In ovules where three sectors are formed, the zygotic sector can out-compete the two haploid sectors and dominate the shoot apical meristem. This will also result in the loss of the haploid/chimera producing phenotype. However, all the ovules we observed were scored before the first division, and therefore before the phenotype could be lost or concealed. This resulted in higher *Semigamy* expression levels and more accurate differentiation between the degree of phenotype manifestation in all types of crosses.

However, though the levels of *Semigamy* expression we detected were significantly higher than those reported elsewhere, our data also show that, in accordance with previous literature, *Semigamy* is incompletely expressed, as evidenced by the presence of sexual ovules in homozygous crosses (6% of *SeSe* x *SeSe* crosses). This may be the result of an underlying predilection towards sexuality supplemented by various developmental and/or environmental factors untested in our analysis. It would be interesting to use this method to analyze *Semigamy* expression in various diverse environments to see what role environmental interactions could have on its expression.

Interestingly, we also detected low frequencies of developmental states that were cytologically-indistinguishable from the *Semigamy* phenotype in egg cells of plants that did not carry the *Se* mutation (*sese* x *SeSe* 0.03, *sese* x *Sese* 0.02, and *sese* x *sese* 0.01), a finding not previously reported in any earlier studies. The simplest explanation for these events is that these samples were harvested and chemically fixed in the small time frame between syngamy (cell fusion) and synkaryon formation (nuclear fusion). Though non-mutant egg cells progress rapidly through these two events, there is still enough time to capture some intermediary zygotes if one looks at enough samples.

In summation, we used a new semigamy screening method to directly observe reproductive samples to record and study the *Semigamy* mutation in Pima cotton as it occurred. This allowed us to form a more concrete view of how and when the *Semigamy* gene is expressed, which should serve to advance both further basic research on apomixis and the usage of this particular mutation in cotton breeding and reproductive analysis.

CHROMOSOMAL LOCALIZATION OF THE *SEMIGAMY* GENE OF *GOSSYPIUM BARBADENSE* USING AN ANEUPLOID MATING SCHEME

Introduction

Many scientists have theorized that apomixis can benefit both breeders and producers if it could be successfully introduced into certain agriculturally important species. Plants capable of asexual reproduction would be reproductively isolated and thus protected from internal genetic dilution and incapable of contaminating wild species with detrimental or genetically modified traits. In addition, instantaneously true-breeding lines would both improve the speed and efficiency of variety releases. However, these advantages remain theoretical because, to date, no one has been able to successfully initiate apomictic reproduction in a sexually reproducing species (Bhat et al. 2005). A variety of different techniques has been attempted, including traditional plant breeding (Savidan 2000), forward (Grimanelli et al. 2001; Ohad et al. 1996, Consiglio 2004), and reverse genetics (Albertini 2005). The many individual evolutions and diverse forms of apomixis imply there should be many ways to develop d*e novo* systems of asexual reproduction, yet this overall goal remains elusive.

Our research focuses on a form of apomixis which in the past has not featured prominently in the field. Since its discovery in 1963 (Tucotte and Feaster), the *Semigamy* mutation from *Gossypium barbadense* (Pima cotton) has proven useful to cotton breeders and researchers but has been overlooked by the apomixis community. Semigamy is a naturally occurring form of apomixis wherein after fertilization of the egg cell (syngamy) the egg and sperm nuclei remain separate and unfused. It has been

documented in a variety of apomictic species, including *Rudbeckia* (Battaglia 1945), *Cooperia* (Coe 1953), and *Zephyranthes* (Crane 1978), and, due to the difficulties inherent in temporally location and observing karyogamy in plants, it may be present in many more (Gwyn 1995). In most semigamous species the paternally derived cells present after the first few mitotic divisions are developmentally isolated, but with the *Semigamy* mutation in cotton paternally derived cells often develop alongside their maternal counterparts, resulting in the production of chimeric seedlings and plants. In addition, Hodnett (2006) observed the formation of restitution zygotic nuclei at the first mitotic division, resulting in either the loss of the semigamy phenotype or the formation of trichimeric embryos.

Cotton breeders and researchers have been able to utilize the *Semigamy* mutation in several different ways. It has been used to generate homozygous lines (Mahill et al. 1984), to create mapping populations (Zhang et al. 2002), and to study interactions between different cellular layers (Dolan and Poethig 1998). However, the actual mechanism underlying the *Semigamy* mutation remains unexplored, and this limits its usefulness. In addition, the genetic irregularity that causes the semigamy phenotype affects an unknown and uncharacterized cellular process that acts at the most basic levels of plant reproduction. If researchers could elucidate the way(s) in which this process is affected, they could eventually determine its natural functioning and, ideally, ways to further manipulate it in beneficial and useful means.

Localizing the *Semigamy* mutation to a specific region of the genome would only increase its utility and eventually aid in determining the root causes of the *Semigamy*

phenotype. Localizing the mutation would also lead to the mapping and eventually cloning the gene, which would help identify the protein(s) and pathways involved in karyogamy. Even in the short term, localizing the gene to within the close proximity of a well-characterized genetic marker would enable earlier and faster screening of segregating populations, as one could genotype the plants before they reach reproductive maturity. Localizing the mutation to a particular arm would also enable researchers to use hypoaneuploid and hyperaneuploid stocks to characterize the activity of the *Semigamy* mutation at various ploidy levels. This would clear up much of the current confusion over whether the *Semigamy* gene acts recessively in the newly formed zygote or whether it acts recessively or dominantly in the male and female gametes.

To this end, we backcrossed homozygous *Se* mutant lines from *Gossypium barbadense* to a *Gossypium hirsutum* aneuploid population. We hoped to localize the *Semigamy* mutation onto a particular chromosomal segment by analyzing the segregation patterns produced by the various aneuploid plants.

Materials and Methods

Population Development

For this study we used a modified version of techniques that had previously been used to map cotton mutations with aneuploid lines (Kohel et al., 2002; Gwyn, 1995). A population of monosomic, monotelodisomic, and translocation-derived duplication deficient *Gossypium hirsutum* plants are maintained by Dr. David Stelly and Wayne Raska of the Cotton Cytogenetics Lab, Texas A&M University, College Station, TX. A subset of this population was selected that provided unique coverage of approximately

50% of the cotton genome. These individuals were used as females in crosses with a line of homozygous semigamous mutant (line 57-4 provided by Dr. Mac Stewart, University of Arkansas, Fayetteville, AR). The resulting F1 individuals were screened for the maternal aneuploid condition using both phenotypic and meiotic evaluation. Aneuploid individuals were used as males in backcrosses with homozygous semigamous females. The BC1F1 individuals produced by this cross were scored for levels of *Semigamy* expression (Figure 3).

Aneuploid Identification

Putative aneuploids were identified based on phenotypic qualities with the help of Mr. Wayne Raska of Texas A&M University, College Station, TX. These early identifications were validated by meiotic bivalent analysis. Pre-meiotic, meiotic, and post-meiotic buds were collected from the plants over a period of several weeks. These buds were fixed in 3:1 ethanol (95%) to acetic acid for at least one week before analysis. A small cluster (3-6) of anthers was collected from each bud and immature meiocytes were manually pressed out of the anthers into an acetocarmine solution. The meiocytes were then examined under phase contrast to assess their developmental stage. Those that appeared to be at or around metaphase I were heated in solution for one minute at 65^{o}C then squashed under a coverslip using manually applied pressure. The slides were stored at -20^{0}C and analyzed under phase contrast (Figure 4).

Sample Collection and Screening

Fifteen BC1F1 plants derived from each aneuploid were planted in peat pellets in the greenhouse. Ten seedlings from each group (or all germinated seedlings, if less than ten) were transplanted to the field and allowed to flower. Immature flowers were fastened closed with a metal clip one day before flowering and harvested four days later. Ovules were collected from these flowers and immediately immersed in FAA_{50} (35% water, 50% ethanol (95%), 5% glacial acetic acid, 10% formalin). Any plants that were haploid (based on phenotypic evaluation—small leaves and flowers, "zigzag" branches, and infertility) were automatically scored as being homozygous for the *Se* mutation.

Ovule Processing

Ovules were dissected and processed as described previously (Hodnett et al. 1997; above). Briefly, ovules were fixed for at least twenty-four hours in FAA_{50}. The inner and outer integuments were then removed, and the nucelli hydrated before being stained overnight in 0.8% Azure C in 0.05 M sodium phosphate buffer (pH 7.0). Samples are dehydrated, cleared in methyl salycilate, then analyzed using a 63 x Neofluar objective (N.A. = 1.25, W. D. = 0.5 mm) kindly provided by Dr. Spencer Johnston, Texas A&M University, College Station, TX, on a Zeiss Universal II microscope.

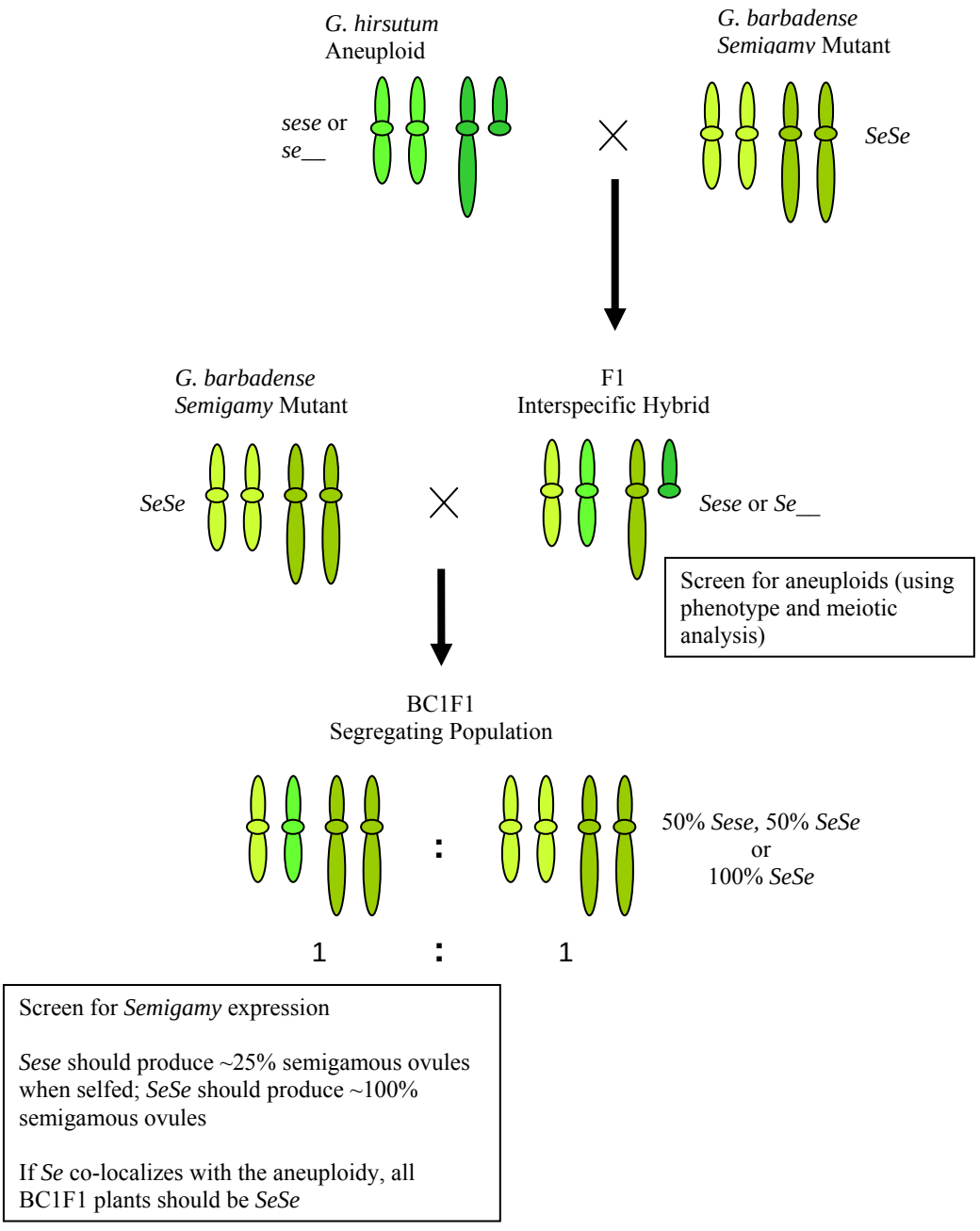


Figure 3. Outline of Aneuploid x *SeSe* Mutant Mapping System.

The above scheme generates segregating BC1F1 *G. hirsutum* x *G. barbadense* populations that can be screened for the *Semigamy* phenotype. The trait should not segregate in the one population created from the plant hemizygous for the trait. Note: both of the crosses diagramed above are written female x male.

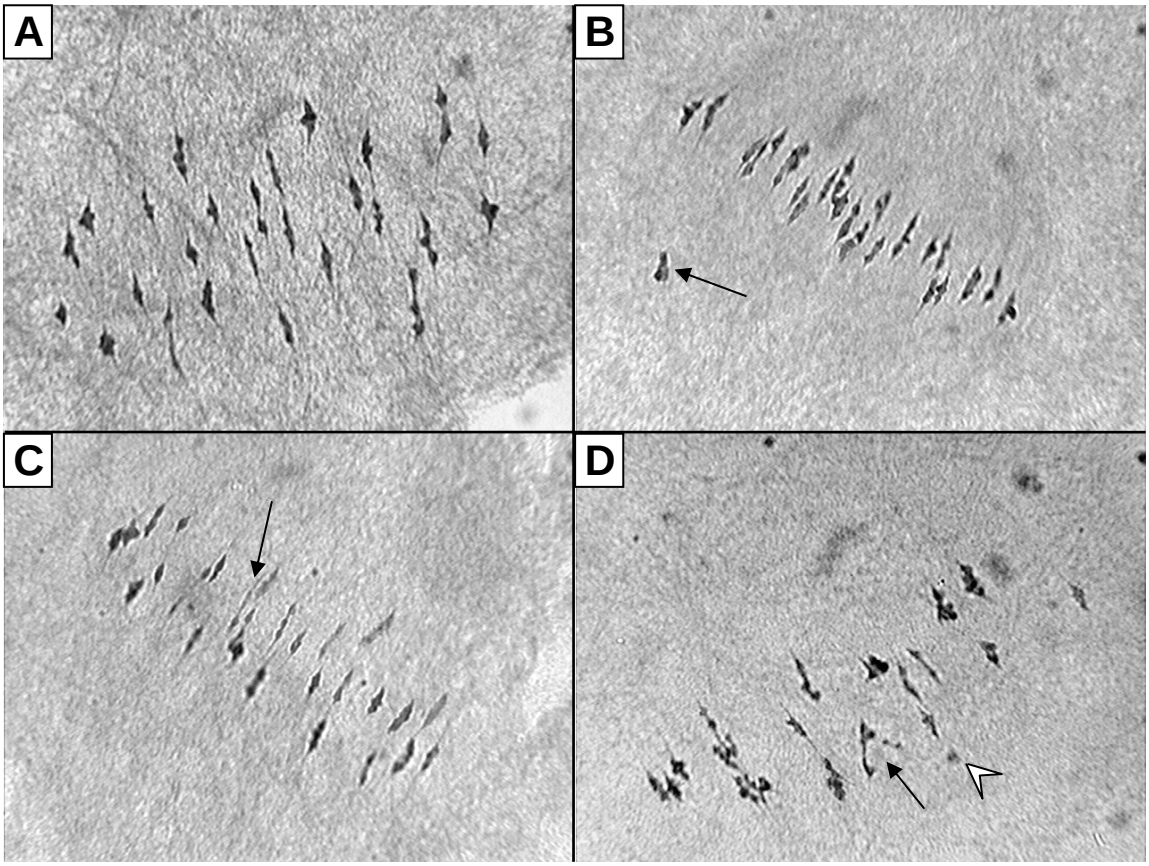


Figure 4. Examples of Meiotic Spreads from Various Aneuploids.

(A) Normal chromosome complement (26II) from a *G. hirsutum x G. barbadense* hybrid (2n = 52). There are 26 bivalents. (B) Chromosome spread from a *G. hirsutum x G. barbadense* hybrid monosomic for chromosome 12 (2n = 51). Univalent is marked by an arrow, and there are only 25 bivalents. (C) Chromosome spread from a monotelodisomic plant (2n = 52) missing the long arm of chromosome 22. Abnormal bivalent is marked by an arrow. (D) Chromosome spread from a translocation-derived segmental trisomic monosomic plant (2n = 52) that carries a duplication of a segment of chromosome 12 and is deficient for a segment of chromosome 19. This has resulted in this chromosome set containing 24 bivalents, one trivalent (marked by an arrow), and one univalent (arrowhead).

Results and Discussion

In the absence of aneuploidy, one would expect the BC1F1 population outlined above to segregate 1:1 heterozygous : homozygous plants at all genetic loci. However, because the hypo-aneuploid condition (e.g. monosomy) cannot be transmitted through the male gamete (Endrizzi et al. 1985), F1 plants hemizygous for the *Semigamy* mutation can only transmit the mutant allele to their paternal offspring. In other words, BC1F1 individuals derived from plants missing the chromosomal segment that carries the *Semigamy* gene will segregate 0:1 heterozygous : homozygous mutant plants. Heterozygous (*Sese*) and homozygous (*SeSe*) plants can be differentiated based on the levels of expression of the *Semigamy* phenotype. After selfing a heterozygous plant will produce ~25% semigamous offspring, while a homozygous mutant individual will produce 90-100% semigamous offspring. Using these differing frequencies we were able to determine the genotypes of all tetraploid BC1F1 plants.

A number of plants in the backcross population were haploid and/or chimeric. This is to be expected in a population derived from homozygous semigamous mutants, but the genotype of these plants could not be determined using the procedure outline above because the haploid plants did not produce viable gametes. Instead, all haploid plants were scored as homozygous mutant. The maternal parent of the plants was a homozygous mutant, and the paternal parent was either heterozygous or hemizygous for the mutation. Previous studies (Turcotte and Feaster 1974, Zhang et al. 1999, this work) have shown that *SeSe* x *Sese* crosses produce progeny that segregate 1:1 semigamous : wild type. Therefore, we can concluded that only the offspring derived from two

gametes that both carry mutated *Semigamy* genes (i.e. *SeSe* zygotes) express the *Semigamy* phenotype and therefore have the potential to become haploid or chimeric plants. However, because there is a demonstrable maternal effect when a homozygous mutant female is crossed with a wild-type male, a small portion of the haploid individuals may in fact be derived from heterozygous gametes. This small subset of mis-scored individuals will not irrevocably alter our results as both types of BC1F1 populations (segregating and non-segregating) are expected to produce homozygous mutants. Rather, differentiating the two types depends on the presence or absence of heterozygous plants. Most, probably all, of the heterozygous plants will be tetraploid and will therefore be discovered through ovule screening.

We screened several BC1F1 populations derived from individual monosomic, monotelodisomic, and duplication-deficient plants in order to determine their relative segregation pattern. Only those aneuploids that produced non-segregating populations were considered to be of interest; therefore, if more than two heterozygous individuals were found in a single population the entire population was considered to be segregating. Figure 5 shows the resulting segregation patterns for each population. All of the tested populations segregated for the *Semigamy* trait, indicating that it is not present on any of the chromosomal segments included in our study. Though this result was not the most desirable outcome of this experiment, it does eliminate approximately 58% of the cotton genome (30 out of 52 chromosomal arms). This knowledge will aid in future efforts to localize the gene using more cotton aneuploids and/or molecular marker based approaches.

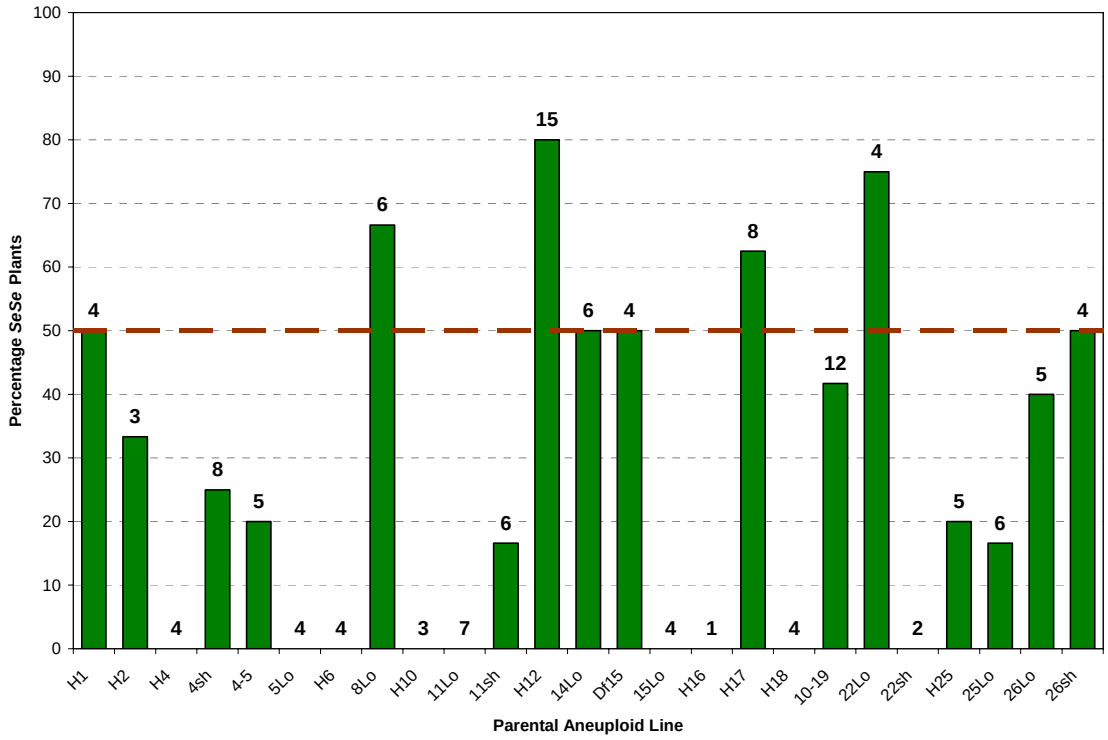


Figure 5. Quantification of Segregation in BC1F1 Populations from Various Aneuploid *G. hirsutum* x *SeSe G. barbadense* Lines.

The numbers above each bar indicate the number of individual plants screened from that population.

Interestingly, the overall frequency of the *SeSe* genotype was lower than expected. We screened 134 individuals from 25 different aneuploids. As all the aneuploids produced segregating BC1F1 populations, we would expect that 50% of the 124 individuals (i.e. 67 individuals) would be homozygous for the *Semigamy* mutation. Actually, one could argue that the percentage of *SeSe* plants should be higher than 50% because, as discussed previously, all haploid plants were scored as *SeSe* even though

there was no definitive way to determine their genotype. The observed *SeSe* frequency of 0.35 is statistically significant from the expected 0.5 ($\chi^2_{CALC} = 11.95$, $p < 0.001$ with one degree of freedom). This could indicate some form of selectional bias against *Se* gametes or *SeSe* homozygous plants. Possible sources of this bias would include deleterious interactions with *G. hirsutum* genes, as the *Semigamy* was discovered in *G. barbadense* and has been predominately studied in that background. However, previous crosses between semigamous lines and *G. hirsutum* populations have not been seen to have an effect on *Semigamy* expression (Gwyn 1995). Alternatively, the frequency could have been lowered if there was a bias against haploid plants when the plants were transplanted to the field from the greenhouse.

Although this project did not directly localize the *Se* gene to a specific chromosomal arm or segment, the resulting information and plants will be valuable in subsequent research. The cytogenetic test results eliminate 58% of the cotton genome from future localization efforts and will thus guide future gene localization efforts and render them more efficient. We now have a segregating *G. hirsutum* x *G. barbadense* mapping population in which every individual has been evaluated for the *Se* phenotype. Even though it was created using various aneuploid parents, this population can still be used in a molecular marker-based approach for the localizing of the gene. In short, though it may not have yielded all we desired, the work potentiates and fosters subsequent sucess.

A REVERSE-GENETICS APPROACH TOWARDS THE GENERATION OF APOMICTIC REPRODUCTION IN *ARABIDOPSIS THALIANA*

Introduction

The efforts to introduce apomixis, or asexual reproduction through seed, into agriculturally important crop species have taken many forms, from traditional plant breeding to gene cloning to mutant screens (Bhat et al. 2005). All of these have resulted in significant scientific advances but produced no newly asexual species. However, the recent advent of effectual genomic sequencing, and the resulting array of whole-genome, transcriptome, and proteomic methodologies, has generated a host of novel tools and procedures that can be applied to this problem. In this newly genomic world, the model species *Arabidopsis thaliana* is gaining prominence as a useful tool for the study and *de novo* generation of apomixis in a sexually reproducing species.

Since the completion of the *Arabidopsis* sequencing project in 2000 (Arabidopsis Genome Initiative 2000) and the onset of the Arabidopsis 2010 Project, many resources have become available to the entire *Arabidopsis* community. These include an online, annotated, searchable genomic sequence; a database of published microarray results for thousands of genes; libraries of characterized *Arabidopsis* ecotypes and mutants; facilities for creating and/or identifying mutations in particular genes of interest; a database of sequenced and localized molecular markers of all types (RFLPs, SSR, SNPs, etc.), and collections of full-length cDNAs collected at various developmental stages and environmental conditions (Ausubel 2002). These resources enable researchers to analyze and use this species in ways unimaginable in other plant species.

The above advances enabled the introduction of reverse genetics based inquiries into the study of plant genetics. Reverse genetics involves the identification of candidate genes based on various factors, e.g. expression profile or sequence homology. Researchers then obtain and analyze the phenotype of any available mutations in these genes. This approach is more amenable to the analysis of certain biological functions, e.g. plant reproduction, than traditional forward-genetic methods. This is partly because it is difficult to screen for small alterations in the various processes involved in plant reproduction. Most studies have been done on male gametogenesis due to the ease in obtaining large numbers of pollen mother cells and microgametophytes (Yang et al. 2003, Park et al., 2004). However, there is substantial evidence that male and female reproduction uses different genes (Yang and Sunderasan 2000). Studies of mega-gametophyte development are hampered by several factors. The female gametophyte consists of only seven cells that are buried within several layers of protective tissue. The events of pollination, fertilization, and embryo formation progress very rapidly with few external landmarks to denote their passing. Small alternations cannot be effectively identified with the speed and accuracy necessary for large-scale mutant screens. As a result, most documented mutations affecting female fertility result in either complete loss of fertility or embryo lethality.

In contrast, meiosis, and many alterations thereof, are well characterized in several model species, and reverse-genetics techniques allow researchers to take advantage of this information. Several studies have noted that there is significant homology between *Arabidopsis* genes and those of other model organisms, including

yeast, fruit fly, and humans, and several labs are beginning to take advantage of this fact (Mercier et al. 2001, Stevens et al. 2004). However, mining for potentially helpful mutations in other organisms begins with defining exactly what these mutations should look like.

To replicate apomictic reproduction in a sexual species like *Arabidopsis,* one could begin by looking for single mutations that comprehensively confer virile asexual reproduction. However, these types of options are limited, as no definitive "apomixis gene" has ever been cloned. In fact, the research strongly indicates that the asexual reproduction requires complex manipulations of multiple genes (Bicknell and Koltunow 2004). Instead, it would be more logical to begin by subdividing apomixis into its component operations and looking at various ways those tasks could be accomplished. Apomixis consists of three parts—generation of a doubled egg cell, prevention of paternal genomic contribution (fertilization), and parthenogenesis (Koltunow and Grossniklaus 2003). There are multiple mechanisms that apomictic plants employ to accomplish these tasks (Crane 2001, Savidan 2000). There are also mutants in other organisms that emulate these processes. For example, in *S. pombe* the *spo12* and *spo13* mutant strains produce diploid spores (Klaphotz and Esposito 1980), and various *S. cerevisiae* mutants are incapable of nuclear fusion (Miller and Rose 1988). The *c-mos* mutation in mice allows for parthenogenic reproduction (Colledge et al. 1994). Mutations in the *ff16* gene of *Drosophila* result in a lack of cytokinesis at the MI stage, which can sometimes produce unreduced gametes (Feborova et al. 2001), while mutation in *gld1* and *gld2* of *C. elegans* force the germline cells to forgo meiosis entirely and

instead undergo mitotic divisions (Kadyk and Kimble 1998). It is important to keep an open mind when looking for parallels of any given phenotype in other species. The given characteristics of any phenotype will be altered, for better or worse, by several factors, including the naturally occurring divergent evolution of homologous genes and the altered biology of *Arabidopsis* as compared to the original organism. To expect identical phenotypes from homologous genes all the time is unreasonable, but one can judiciously hope that most of the homologous genes identified would at least be involved in the same basic processes.

Lastly, several genome-wide analyses in various species have uncovered a variety of sequenced but uncharacterized genes that could also be involved in apomictic reproduction (Barcaccia et al. 2001, Albertini et al. 2004). Some researchers have examined the effect of overexpressing these candidate genes in *Arabidopsis*, with variable results (Kantama et al. 2006). The genes and sequences discovered in these studies and others like them would also make good initial bait for homology searches.

In this study we identified mutations from several different species with phenotypes that mimic some part or form of apomictic reproduction. This database of interesting mutants was compared to the database of known *Arabidopsis* proteins, allowing for the identification of homologous genes. *Arabidopsis* mutant lines containing T-DNA insertions in these genes of interest were obtained from the Salk Institute for Genomic Analysis (SIGnAL, Alosnso et al. 2003). These lines were briefly characterized for any physical or reproductive abnormalities. Eventually we hope to develop a collection of novel *Arabidopsis* reproductive mutants. Individually, these

mutants could be used to elucidate the basic processes and genetic regulation of sexual plant reproduction. In combination, perhaps, these mutants could eventually be used to recreate a viable form of asexual reproduction in a sexual species.

Materials and Methods

Arabidopsis in silico *Analysis*

Cloned genes or cDNAs considered "of interest" for their role in apomixis, meiosis, parthenogenesis, karyogamy, or asexual reproduction were culled from published papers and internet databases. These sequences were compared to the entire database of *Arabidopsis* proteins using NCBI's protein-protein BLAST (blastp) (Altschul et al. 1997). BLAST results were evaluated on an individual basis using the following rough guidelines: Scores >200 or e-values <1e-10 were considered of special interest, but not if more than ten matches had equally high scores. In genes returning less than three matches, all were considered significant no matter the scores or e-values. Lastly, long (>1/3 of protein length) matches were considered to be of interest in spite of their score. Matching protein sequences were searched against the *Arabidopsis* genome using the SIGnAL "T-DNA Express" *Arabidopsis* gene mapping tool (Alonso 2003). Matches carrying a T-DNA insertion in their coding regions were ordered from the Arabidopsis Biological Resource Center.

T-DNA Homozygous Identification

The SIGnAL T-DNA primer design program was used to identify lines homozygous for the T-DNA insertion. PCR primers were designed for each line that flanked the proposed sight of insertion by ~450 bp on either side. We also used an

"Insert primer" which was specific for the left border sequence of the T-DNA insertion (3'-AGTTGCAGCAAGCGGTCCACGC-5'). Using this system, wild-type lines yielded an approximately 900 bp fragment while homozygous mutant lines will gave smaller, ~400 bp fragments (depending on how much of the left border sequence was inserted), and heterozygotes yielded two distinct fragments. The progeny of lines with obvious physical phenotypes were scored to ensure co-segregation of the T-DNA insertion and the phenotype.

Arabidopsis *Cytology*

Siliques, flowers, buds, and floral meristems were collected from *Arabidopsis* flowers at various stages of development. The samples were fixed in FAA_{50} (50% Ethanol (95%), 35% water, 10% formalin, 5% glacial acetic acid) until they sunk to the bottom of the vial (usually 2-3 days). The samples were then hydrated (50% ethanol in water, 25% ethanol in water, then two rinses of pure water, 30 minutes each) and stained overnight with 0.08% Azure C in 0.05 M sodium phosphate buffer (pH 7.0). Afterward the samples were dehydrated in 10% increments of ethanol in water (15 minutes each) before being cleared in methyl benzoate (5:1, 2:1, 1:1, 1:2, 5:1, 0: 1, 0:1 ethanol : methyl benzoate, 22 minutes each). Ovules were manually removed from their siliques using sharpened embroidery needles and then analyzed on a Zeiss Universal II microscope using DIC filters with a 63x Neofluar objective (N.A. = 1.25, W. D. = 0.5 mm) kindly provided by Dr. Spencer Johnston, Texas A&M University, College Station, TX. Images were obtained through an Optronics VI-470 CCD camera (470 line, RGB/grayscale) attached to a 0.7-7 x zoom lens system. Digital images were produced

by averaging eight still shots and were adjusted for brightness and contrast using both camera controls and, in some cases, Adobe Photoshop™ software.

Transmission Deficiency Assay

Homozygous mutants were used as females in crosses to wild-type plants. The F1 plants were then reciprocally crossed to wild-type plants, yielding back-cross populations that each contained over 100 plants. This population was scored for transmission of the T-DNA insertion using the PCR-based screening procedure discussed above.

Results

In silico *Analyses*

Our research began with the creation of a database containing various "mutations of interest." These were mutations from any species—plant, animal, fungal, or microbial—that had a phenotype that mimicked some form or aspect of apomixis. We also incorporated the results from genomic comparisons between apomictic individuals and their closely related sexual relatives, including results from cDNA-AFLP comparisons in *Medicago* (Barcaccia et al, 2002) and differential display in *Pennisetum* (Vielle-Calzada et al, 1996). A complete list of the identified genes of interest is provided in Appendix A. A total of 267 genes of interest were identified. Most of these genes (51.9%) were originally discovered in S. *cerevisiae* (Figure 5), which testifies to how well characterized this organism is with respect to meiotic and reproductive mutations. The second highest proportion of mutants was derived from *M. sativa* (13.2%), due to a comprehensive and well-published cDNA-AFLP comparisons between

wild-type alfalfa plants and an apomeitoic mutant which produces large numbers of 2n egg cells (Barcaccia *et al*, 2002). Large numbers of mutations were also identified in *S. pombe* (9.4%) and *Drosophila* (9.0%), which are both widely used model species that have undergone many genetic screens for reproductive abnormalities.

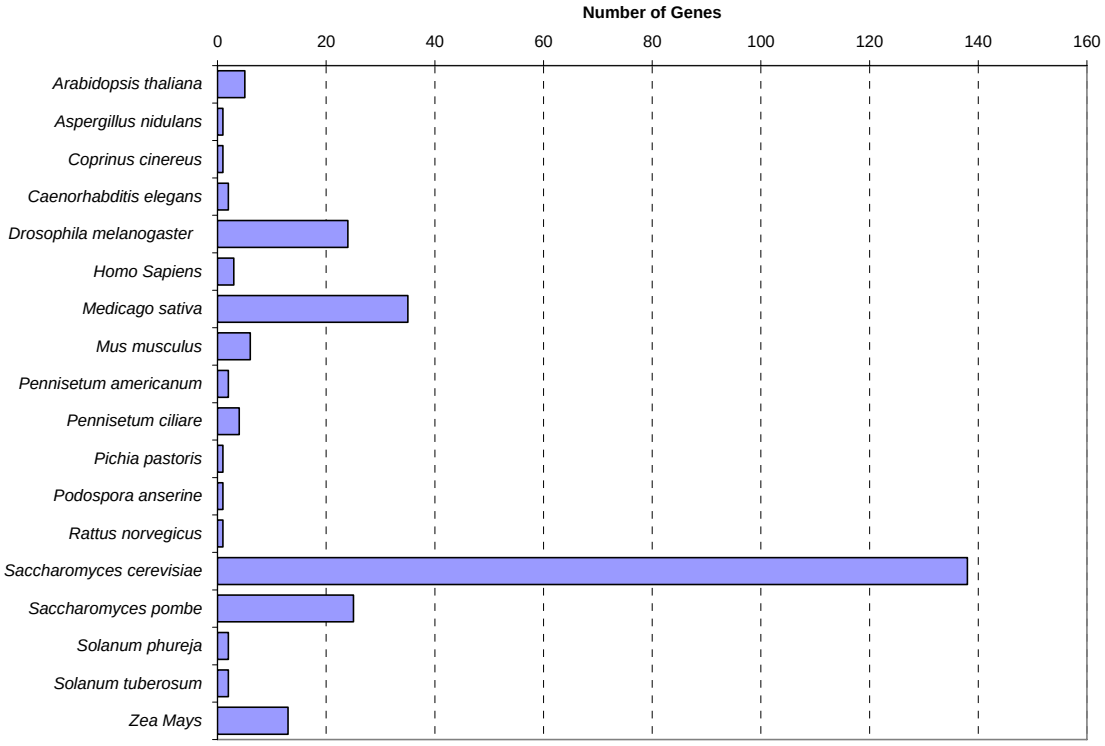


Figure 6. Distribution of "Genes of Interest" by Species of Origin.

In most instances, mutations in these genes produced phenotypes that mimic some form of apomixis. The genes for *Medicago sativa* and *Pennisetum ciliare* are from whole-genome expression comparisons between apomictic and sexual plants.

Of the 267 mutations of interest, only 209 had nucleotide or protein sequences available in the public database. Unfortunately, many of the mutations discovered in plant species (*Z. mays, S. phureja,* and *S. tuberosum*) are in genes that have yet to be cloned or sequenced. In addition, the cDNAs discovered in *Pennisetum ciliare* proved to be too short for accurate genome-wide comparisons. However, most of the mutations discovered in the model species had long and presumably accurate nucleotide and protein sequences available in NCBI's Entrez nucleotides and protein databases. For the purpose of this study we chose to use NCBI's protein-protein (blastp) program (Altschul et al, 1997) to compare the available sequences to the entire database of available *Arabidopsis* protein sequences. A protein-protein search was chosen in the belief that it would help eliminate problems with codon bias or naturally occurring non-effective mutations arising through centuries of divergent evolution. In the cases where protein sequences were unavailable (e.g. the cDNA-AFLP results from Barcaccia *et al* (2002)), a blastx query was used (Altschul et al. 1997), which translates a nucleotide search string in all six reading frames then compares each result against the same *Arabidopsis* protein databases used during a blastp search.

Ideally, each blastp search would have returned a small number (<3) of unique matches with high scores and low E-values. Some queries did match this formula and their results were recorded in the appropriate databases. Examples of this include *cin8*, which had only two matches, both of which were near full-length, and *mnd1*, which had only one. Both of these were from mutations originally discovered in *S. cerevisiae.* However, this type of result was unfortunately quite uncommon. In order to effectively

screen all the protein matches and identify as many potential homologous sequences as possible, no definite "cut-offs" were established for score or E-values. Instead, each result was evaluated individually based on several criteria. The most abundant type of match returned by the blastp search was a long list (50 to >200) of matches that was usually generated because the original sequence contained well-conserved domains that define large families of proteins. Examples of genes that returned this type of match include *cdc28* from *S. cerevisiae*, which is a serine/threonine protein kinase, and *arl2* from *H. sapiens*, which is a RAB/RAS GTP-binding protein. In these cases the highest scoring match was documented along with any unusually long matches or matches that were homologous to regions outside of the conserved domain(s). If possible the areas from the original protein sequence that were outside the conserved domain(s) were BLASTed in a separate search. If this second search returned long or high scoring matches, the resulting proteins were also recorded. Lastly, several protein sequences were only homologous to a collection of short, low-scoring sequences randomly arrayed throughout its length (e.g. ccz1 and gip1 from *S. cerevisiae* and spo6 from *S. pombe*). Most often in these cases, none of the matches was considered significant.

All proteins that were identified as having potentially significant homology were reciprocally BLASTed back against the database of known proteins from the species of the original mutation. This reciprocal BLAST was expected to return the original protein as the best match from the database, or, at the very least, the original protein should have appeared very high on a list of homologous matches. If the reciprocal search did not demonstrate significant homology to the original gene, that protein was

removed from the database of interesting *Arabidopsis* proteins.

Lastly, all remaining *Arabidopsis* proteins of interest were BLASTed against the *Arabidopsis* genome using the SIGnAL "T-DNA Express" Arabidopsis Gene Mapping Tool. This allowed us to identify *Arabidopsis* lines that carried T-DNA insertional mutations in genes that code for these proteins of interest. Of the approximately 150 *Arabidopsis* proteins that were found to be homologous to the original 267 genes of interest, 14 matches to SALK insertion lines were identified (Table 7). Thirteen of these mutants were homologous to *S. cerevisiae* genes, while one was homologous to *rec8* from *S. pombe*. In addition, *cdc48* and *bim1a* were homologous to two separate *Arabidopsis* genes each, herein differentiated as *BIM1a* (*At5g67270.1*), *BIM1b* (*At5g62500.1*), *CDC48a* (*At3g09840.1*), and *CDC48b* (*At3g53230*).

Table 7. Potentially Interesting *Arabidopsis* Genes Carrying Insertions Identified by SIGnAL

At Gene	At Gene function	Original Gene	Organism	Gene Function
At5g67270.1	unknown	*bim1*	*S. cerevisiae*	Microtubule binding protein. Mutants do not undergo karyogamy.
At5g62500.1	microtubule-associated EB1-like protein	*bim1*	*S. cerevisiae*	Microtubule binding protein. Mutants do not undergo karyogamy.
At2g31620	putative receptor kinase	*cdc37*	*S. cerevisiae*	Mutant strains are karyogamy deficient.
At3g09840.1	putative transitional ER ATPase	*cdc48*	*S. cerevisiae*	This cell cycle protein is required karyogamy and peroxisome functioning.
At3g53230	unknown	*cdc48*	*S. cerevisiae*	This cell cycle protein is required karyogamy and peroxisome functioning.
At3g60740.1	beta-tubulin cofactor-like protein	*cin1*	*S. cerevisiae*	A microtubule-associated protein necessary for karyogamy.
At4g09980	unknown	*kar4*	*S. cerevisiae*	Protein known to regulate expression of several genes involved in karyogamy.
At5g19660.1	subtilisin-like protein	*kex2*	*S. cerevisiae*	Mutants arrest between MI and MII of meiosis
At5g16270.1	unknown	*rec8*	*S. pombe*	Mutants undergo equational instead of reductional division at MI and sometimes produce diploid asci.
At5g25070.1	unknown	*spc42*	*S. cerevisiae*	Mutants produce asci with two haploid spores.
At5g18930	S-adenosyl-L-methionine decarboxylase - like protein	*spe2*	*S. cerevisiae*	Required for haploidization and sporulation.
At3g05630.1	phospholipase D family protein	*spo14*	*S. cerevisiae*	Required for commitment to meiosis and MII
At5g16210.1	unknown	*spo3*	*S. cerevisiae*	Mutants produce asci with one or two haploid spores.
At3g48430.1	unknown (similar to Zn finger)	*ste12*	*S. cerevisiae*	Functioning gene is required for cell cycle arrest and karyogamy

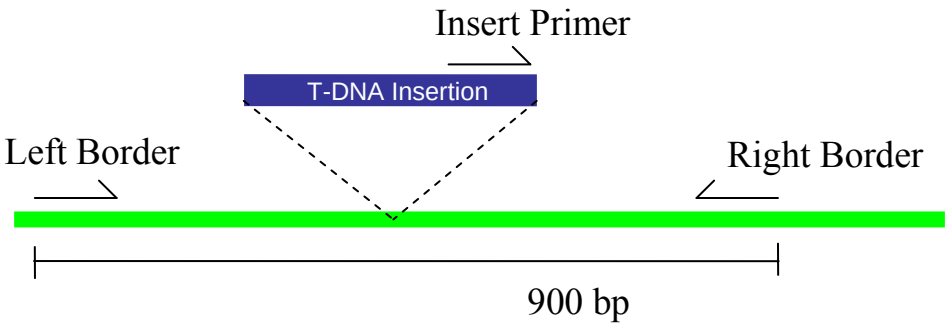
Effect of T-DNA insertion of Gene Expression Levels

Lines homozygous for T-DNA insertions in the genes of interest were identified using primers that flanked each insertion site and a primer from the left border sequence of the T-DNA vector. In wild-type plants the left and right border primers were designed to yield a ~900 bp product. However, in the presence of a T-DNA insertion the left and right primer sequences would fail to amplify and, instead, a much shorter fragment would form that spanned from the left border of the T-DNA insertion to one of the flanking primer sequences from the genome (Figure 7). Whether this occurred in conjunction with the left or right border primer would depend upon the orientation of the T-DNA insertion. This technique was performed on ten T4 generation plants from each mutant line. These lines had been allowed to self four times since the original mutation event and in each generation had undergone selection for the T-DNA insertion. It was expected that all plants would be homozygous for the insertion. However, all lines still had a few (ranging from 1 to 5) heterozygous individuals, and in lines *spe2*, *spo14*, *kex2*, *cdc48a*, and *cdc48b* no homozygous individuals could be identified. Plants from the *rec8* line also yielded interesting PCR results. Plants from this line did not follow the predicted patterns but instead produced one, two, or three PCR products per plant (Figure 7 C&D). It was determined that this was caused by a head-to-head tandem T-DNA insertion event, which is one of several types of nonstandard insertion events that can occur as a result of T-DNA transformation (Nacry et al. 1998). It is possible other types of tandem T-DNA insertions that would be undetectable using the above procedure will have occurred in other mutant lines.

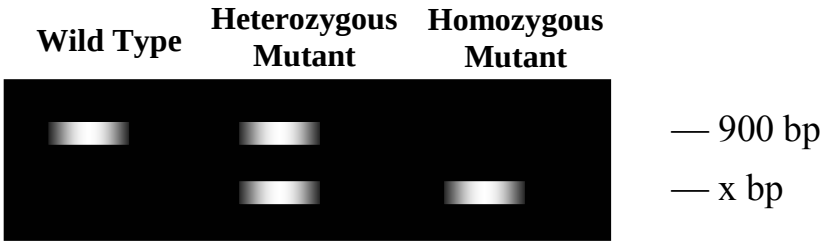
Figure 7. **Schematic of PCR-Based Screening Procedure for T-DNA Insertion.**

(A) Approximate locations of the three primers (left border, right border, insert) in relation to the T-DNA insertion. (B) Expected results from PCR reactions performed on individuals that are wild-type, heterozygous for the T-DNA insertion, and homozygous for the T-DNA insertion. (C) The hypothesized condition of the T-DNA insertion in the *Arabidopsis rec8* gene. In this particular line the T-DNA contains a head-to-head tandem DNA insertion. (D) PCR results from ten T4 individuals from the rec8 population. Lane M contains a ladder, lane 1 a wild-type control. Notes that the individuals in lanes 2, 3, 5, 6, 8, and 10 were homozygous for the T-DNA insertion, lane 9 is heterozygous, and lanes 4, 7, and 11 lack the insertion completely.

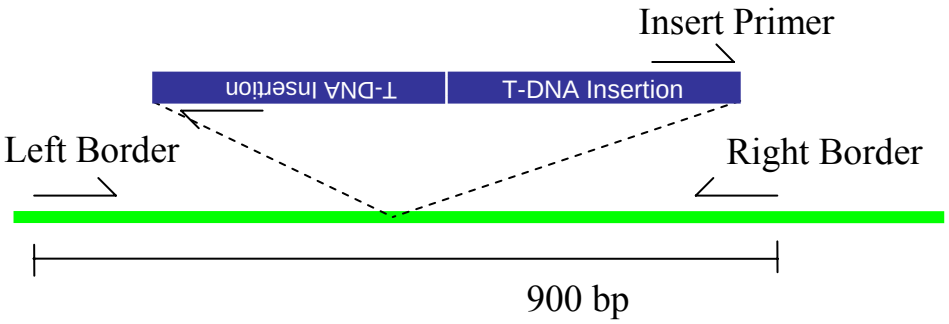
A)


Insert Primer
T-DNA Insertion
Left Border
Right Border
900 bp

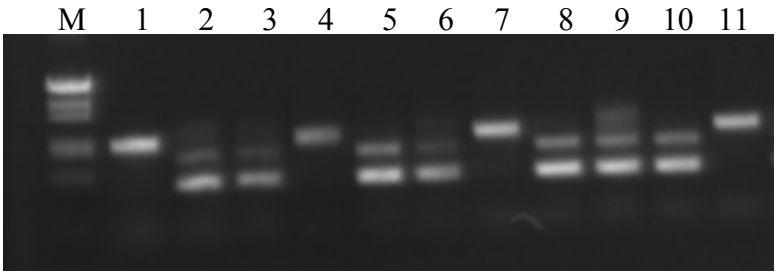
B)


Wild Type
Heterozygous Mutant
Homozygous Mutant
— 900 bp
— x bp

C)


Insert Primer
T-DNA Insertion
T-DNA Insertion
Left Border
Right Border
900 bp

D)


M 1 2 3 4 5 6 7 8 9 10 11

Once homozygous plants were identified, RNA was collected from whole plants and used to determine the effect of the T-DNA insertion on the wild-type expression levels of the genes of interest. RNA was collected from the whole plant because we could not be sure exactly when or where, if at all, the genes would be expressed. By collecting RNA from all tissues of the plant, including young tissue, old tissue, leaves, roots, meristems, flowers, siliques, developing seeds, etc., we hoped to capture enough various expression profiles to virtually ensure the expression of every gene of interest. We also used two different methods to evaluate expression levels of these genes: Northern blotting and RT-PCR. Northern blotting is more quantitative than RT-PCR, yet it is not sensitive enough to detect mRNA expression when it may be confined to a very small number of cells. RT-PCR has the advantage of being more sensitive, but may fail if abnormal splicing variants are produced. As most of the genes we were interested in were putative genes of unknown function, the probability of inaccuracies in the proposed mRNA sequences was quite high. We decided that using both methods would allow us to cover the shortcoming of one with the strengths of the other. The results from the gene expression experiments are summarized in Table 8. In this table "No expression" means the gene was not expressed in the wild-type *Arabidopsis thaliana* controls, "Knockout" means the gene was expressed in the wild-type controls but not in the mutant lines, and "WT levels" means there was no difference in the levels of wild type or mutant gene expression.. We obtained a variety of results, indicating that T-DNA insertion into a specific gene can affect the expression of that gene in a variety of ways.

Table 8. Effects of T-DNA Insertion on Gene Expression Levels

Mutant	Northern	RT-PCR
spc42	WT levels	WT levels
kar4	WT levels	**Knockout**
spo3	no expression	no expression
bim1a	WT levels	**Knockout**
bim1b	WT levels	WT levels
cdc37	**Knockout**	no expression
ste12	WT levels	WT levels
cin1	no expression	no expression
rec8	**Knockout**	**Knockout**

Observable Phenotypes

Two of the mutant lines, *ste12* and *rec8*, demonstrated gross morphological phenotypes that co-segregated with their respective T-DNA insertions. Lines homozygous for T-DNA insertions in the *STE12* gene flowered later and produced generally thicker stems and more upright plants than wild-type plants. We examined the *ste12* phenotype under both long- (24h light) and short-day (8h light) conditions to determine if the phenotype was due to a lack of day length sensitivity (Figure 8). The numbers of leaves produced by *ste12* mutants before the transitions from vegetative to floral meristem (15.7) was significantly higher than that of wild-type (7.4) under long-day conditions. When grown under short-day conditions the number of leaves was also higher for *ste12* (24) than for wild-type (30), though under the difference was not statistically significant. Likewise, under long-day conditions *ste12* required a longer time period before it bolted (58.0 days, as compared to 31.2 for wild-type), but there were no differences between the time to flowering under short-day conditions Under both long- and short-day conditions, the data shows that *ste12* actually grew faster (as measure by leaf production per day) than wild-type plants.

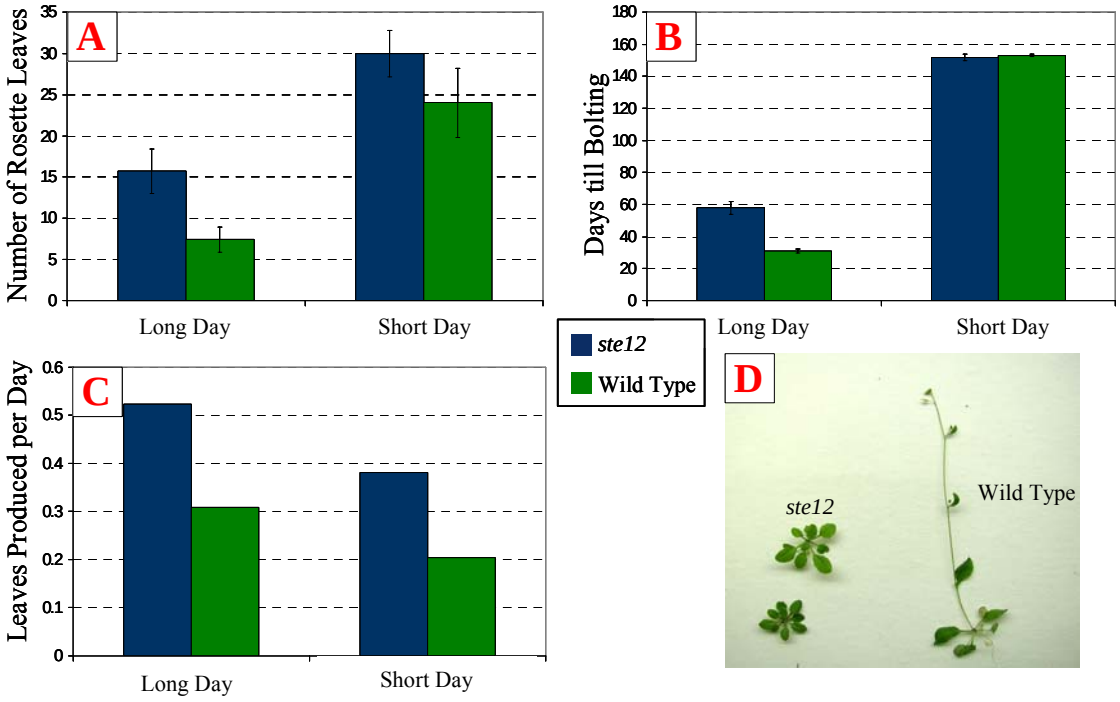


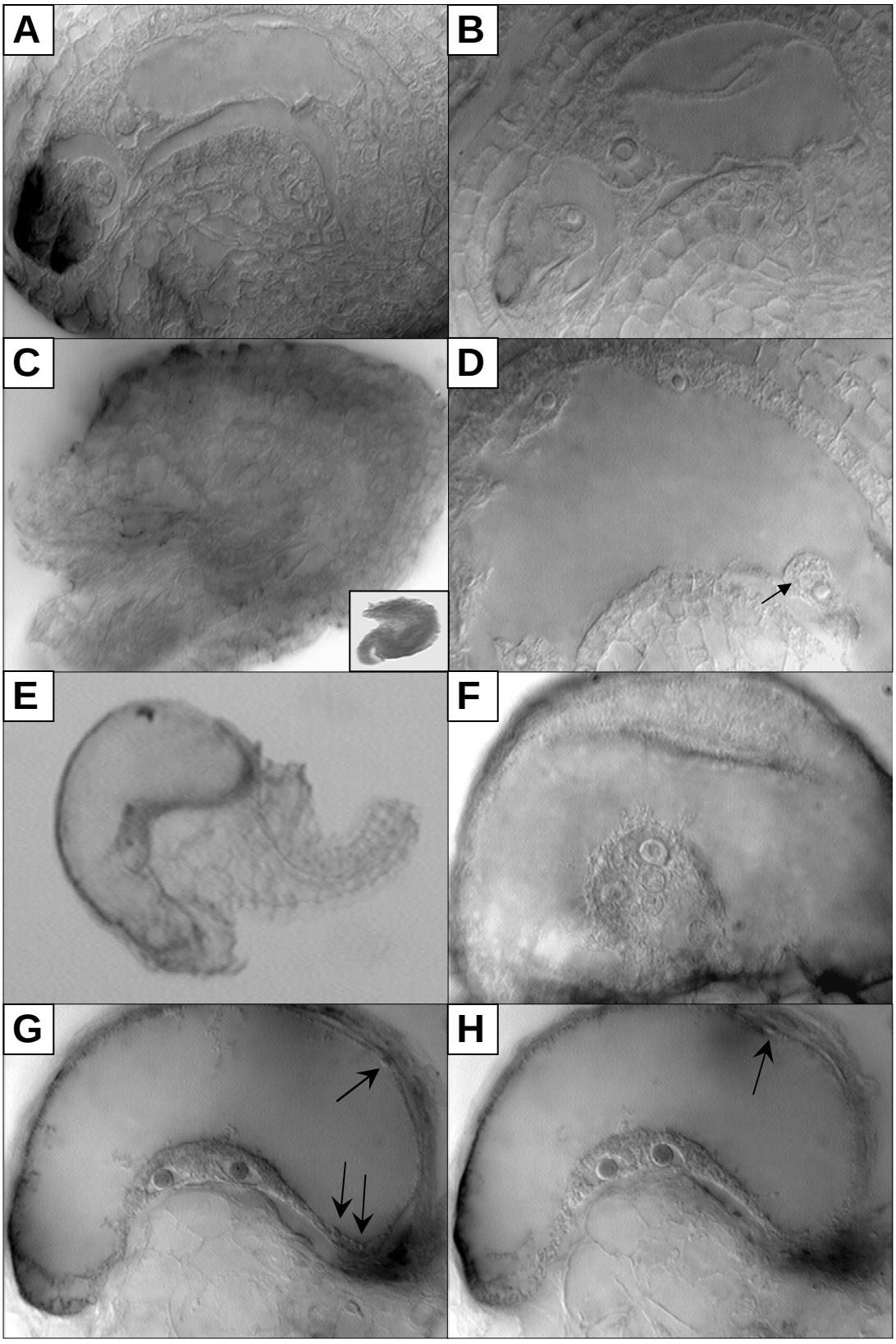
Figure 8. Effects of Day Length on *ste12* and Wild-Type Development.

(A) The number of rosette leaves formed by each *ste12* and wild-type plants before the shift to floral development. (B) A comparison of the number of days between planting and the shift to floral development for each line. (C) A rough comparison of the growth rate for the two lines. Leaves produced per day was calculated by dividing the number of rosette leaves by the number of days between planting and the shift to floral development. (D) A comparison of *ste12* wild-type morphology of young plants (40 days past planting) grown under long-day conditions).

Plants homozygous for mutations in the *REC8* gene displayed spindly growth and reduced fertility. These plants also had smaller seed sets due to a smaller number of siliques. To investigate the cause of this anomaly, *rec8* buds, flowers, and siliques were stained, optically cleared, and viewed with Norma conditions. There were no abnormalities detected during megasporogenesis, the following three rounds of mitosis, or ovule formation. At the time of fertilization, all *rec8* ovules seemed identical in structure to wild-type ovules. However, after fertilization approximately half of the ovules from *rec8* homozygous plants underwent gross morphological malformations (Figure 9). The egg sac of the ovules ballooned outward, distorting the overall shape of the immature seed. The integuments were replaced by a thin acellular band around the egg sacs. The egg cell itself apparently degenerated and could not be positively identified. Instead, one, two, or four very large nuclei were present in the large egg sac. In a small number of ovules, small nucleus-like forms could be detected around the outer edges of the egg sac.

Figure 9. Comparison of *rec8* and Wild-Type Ovule Morphology.

(A) Unfertilized ovule from wild-type plant. (B) Unfertilized ovule from homozygous *rec8* mutant. (C) Example of typical degenerating ovule from wild-type plant. Cause of death is unknown; other ovules in same silique were fertilized and developing normally. Inset is a view of entire ovule (10x magnification). (D) Healthy fertilized ovule from wild-type plant. The undivided zygote is marked by an arrow. (E) Aberrant ovule from homozygous *rec8* mutant under 10x magnification. Note the balloon-like form and lack of integuments. (F) Close-up of an aberrant ovule. There are two large nuclei present in the middle portion of the ovule. There are also two smaller, spherical nuclei present, which are probably the two sperm nuclei. (F&G) Two different planes of focus from the same aberrant *rec8* ovule. This ovule had four large nuclei clumped together in the middle. Note there are also small nucleus-like bodies around the outer edge of the egg sac (arrows). The ovules in D-H were taken from self-pollinated pistils.


A
B
C
D
E
F
G
H

Quantification of Fertility

It is possible that the other mutant lines besides *rec8* and *ste12* have phenotypes that were less obvious. To this end we attempted to quantify the fertility of homozygous mutants from each line by counting the numbers of fertile seeds and infertile seeds contained in individual siliques. We also measured the length of the siliques. The results are summarized in Figure 10. All of the lines displayed varying degrees of reduced fertility compared to wild-type, indicating that either the T-DNA insertion conferring the known mutation or other possible background mutations caused by the transformation process are negatively affecting megagametophyte and embryo viability.

Transmission Deficiency Assay

Transmission deficiency assays are an established means of evaluating the competitive viability of male and female gametes that carry mutant genes over that of normal gametes (Howden et al. 1998, Grini 1999). In brief, heterozygous mutant lines are reciprocally crossed to wild-type plants. The resulting BC1F1 populations are then screened for the presence of the mutation. If the mutation does not affect gamete viability, the heterozygous plants should segregate 1:1 with wild-type plants in the

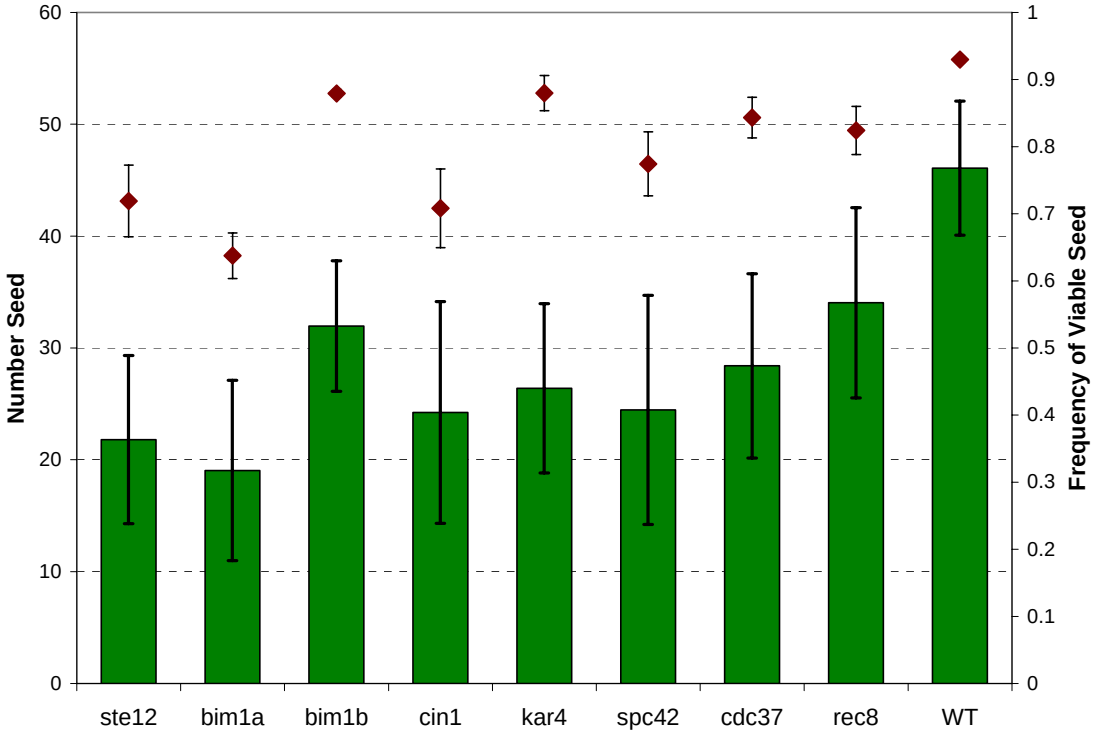


Figure 10. Quantification of Fertility of Homozygous T-DNA Insertion Lines.

The bars show the number of seeds (viable and inviable) per silique for each line (left axis). The line graph compares the average fertility of the various mutant lines, as demonstrated by the frequency of viable seed out of all total seed (right axis). On both graphs the error bars represent the standard error the sample. Sample sizes ranged form 10 to 15 siliques.

population. Populations with ratios less than 1:1 heterozygote : wild-type indicate the mutations deleteriously affect gamete viability, while deviations greater than 1:1 possibly indicate increased viability, possibly through some form apomixis-like reproduction (Figure 11).

We performed a transmission deficiency assay on a subset of our identified mutant lines to assess the suitability of using this assay to characterize all the available mutant lines. Mutant lines *spo3* and *bim1b* were chosen at random to be the first to be investigated. The results from the initial assays are presented in Figure 12. We attempted to genotype 100 individuals for both types of populations (WT x heterozygous (abbreviated het) and het x WT) for both mutants, but variations in plant fertility, PCR success rates, and an unfortunate bout with fungal contamination severely hampered our efforts. The population size is given on top of the bars in Figure 12.

A chi-squared test performed on this data indicated there were some significant deviations from the expected 0.5 frequency for heterozygous individuals in this population. In particular, the T-DNA insertion in the *bim1b* gene greatly impaired female gamete viability (heterozygote frequency = 0.28, χ^2 statistic = 18.98, p-value < 0.001). The *bim1b* mutation also effected male gamete competitiveness, though to a lesser extent than that seen in the female gametes (heterozygote frequency = 0.36, χ^2 statistic = 4.91, 0.01 < p-value < 0.05). The chi-squared tests also revealed that the deviations from 0.5 seen in both of the *spo3* populations were statistically insignificant. When the female parent was heterozygous for the *spo3* mutation 37% of the progeny were heterozygous for the mutation (χ^2 statistic = 2.81, p-value > 0.05), while 45% were

heterozygous when the male gametes carried the mutation (χ^2 statistic = 1.46, p-value > 0.05).

Discussion

In silico *Analyses*

Reproduction is the most fundamental part of biology. All organisms have a fundamental drive to recreate themselves. Sexual reproduction is a corruption of this basic process, as sexually reproducing organisms do not create clonal copies of themselves. However, sex does enable easier genetic recombination and faster evolution, which allows for greater chances of svolutionary success and survival. Sexual reproduction is conserved in some form among nearly all eukaryotic species (Ramesh et al. 2005). The forms, mechanisms, and genetic regulation of sexual reproduction have been studied in a variety of organism, from human to plants to the smallest microbes. This is demonstrated by the wealth of genes and mutations we found in an unavoidably cursory search of the published literature. We discovered 267 mutant genes with phenotypes that mimicked some form of apomictic reproduction or that were differentially expressed in apomictic and sexual relatives. These aided in the identification of 150 homologous *Arabidopsis* proteins. Doubtlessly more homologs to these genes exist, but the difficulty lies in determining the best way to identify functional homologs. In this study we used sequence similarity, an accepted if inconsistent method.

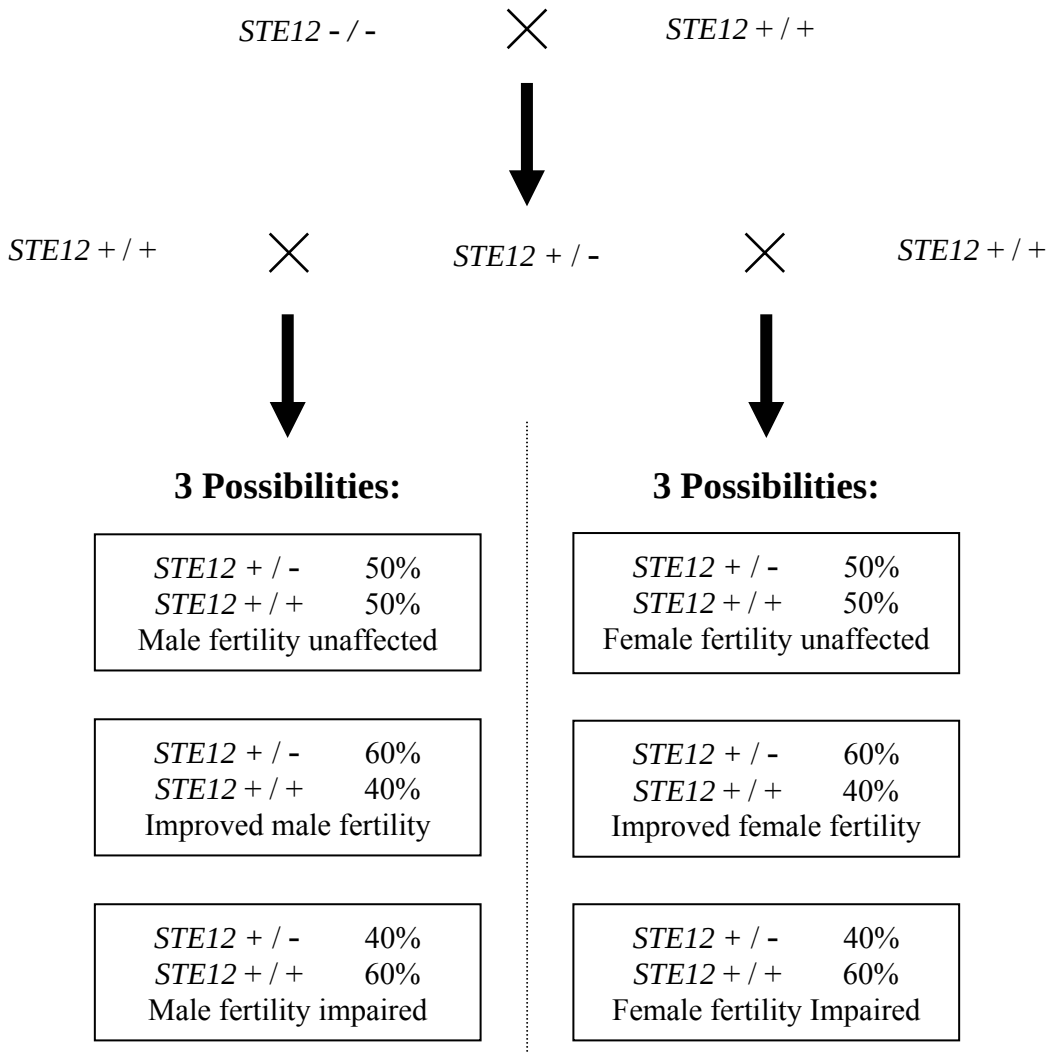


Figure 11. Schematic Outlining the Use of a Transmission Distortion Assay.

Two BC1F1 populations are generated; their only difference being whether wild-type plants are used as the male or female parents. Every cross above is written as female x male. For each population one of three different possibilities can occur—the mutant gametes can out-compete the normal gametes, can be out-competed themselves, or can act perfectly normal themselves. In this figure the line *ste12* is used as an example, but this technique can be applied to all the mutant lines discussed in this study.

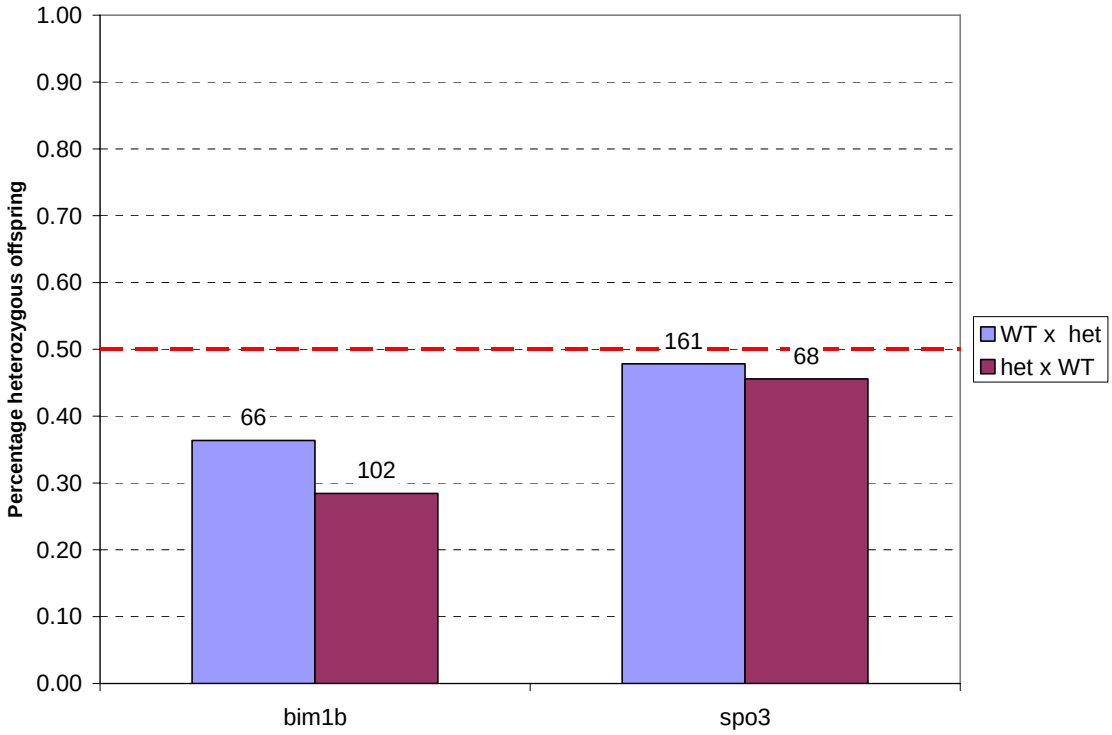


Figure 12. Frequency of Heterozygous Plants in Various BC1F1 *Arabidopsis* Populations.

Transmission Segregation Assays were performed on plants heterozygous for the *spo3* and *bim1b* T-DNA mutations. The frequency of heterozygous plants in recorded by the height of each bar; the number on top of each bar is the total number of plants in that population.

Other techniques exist and could be used in future studies, including complementation analysis with a cDNA library (Bender and Fink 1994). This latter technique would be of especial interest in the yeast species due to their efficient transformation and the relative ease in observation of reproductive mutant phenotypes.

Of the 150 homologous genes identified, 14 were available as T-DNA insertion mutants from the SIGnAL library. This number does not include homologous genes that were identified in our study but are already well studied or characterized by other *Arabidopsis* researchers. The purpose of this study was to identify and characterize new genes and mutants, not repeat what has been done in the past. To that end, all of the *Arabidopsis* mutants we characterized were listed in the database as either of unknown function or as simply belonging to a certain family or class of gene. In addition, several uncharacterized homologous genes had no mutant line available from the SIGnAL library. There are other methods available to obtain mutations in these genes, including TILLING, Ac/Ds mutagenesis, etc (Henikoff et al. 2004, Eto et al 2002). In addition, this number of available mutant lines is continually increasing, both at SIGnAL and in other labs. There is a strong likelihood that mutant forms of all the homologous genes identified in this study will soon be available.

Effect of T-DNA Insertion on Gene Expression Levels

We chose to focus our efforts on homozygous mutant populations in an effort to produce the strongest, most visible mutant phenotypes possible. Our chief fear was the existence of small, possibly incompletely expressed or environmentally affected alterations in the sexual reproduction of *Arabidopsis thaliana,* which would be difficult

to find even in a homozygous mutant population. In addition, embryo lethality was considered an undesirable phenotype because it can be caused by a wide-variety of events and pathway, and successful asexual reproduction depends on the creation of viable progeny. While sterile homozygous mutants would be potentially interesting and useful, dead homozygous mutants would be of little benefit. Unfortunately, in eliminating non-homozygous mutants from most analyses, we eliminated those mutations that could not be transmitted through one type of gamete (male or female), as these would also generate heterozygous but not homozygous mutant lines. However, these particular mutants would be identified in the transmission deficiency assay, which is designed to be performed on heterozygous lines (see below).

The presence of heterozygous individuals after four rounds of selfing implies that these lines are prone to outcrossing and/or gametes carrying the mutation may not be able to adequately compete against their wild-type counterparts. However, we were able to obtain homozygous mutants in most of the lines, indicating a lack of complete embryo or gamete lethality. In can be assumed that in those lines incapable of homozygosity (*cdc48a*, *cdc48b*, *spe2*, *spo14*, and *kex2*) sexual reproduction had been altered in some way. These lines require further analysis to identify the exact nature of their phenotype.

The effects T-DNA insertion had on gene expression varied from none at all to complete loss of expression (Table 8). Only *rec8* showed a loss of expression by both RT-PCR and Northern blot analysis. Transcription of the genes *SPO3* and *CIN1* could not be detected in wild-type plants. This could indicate that these are in fact unexpressed pseudogenes, in which case their mutation should have little effect on any

aspect of plant growth. However, it is also possible that these genes are simply expressed at too low a level to be identified by whole-plant RNA extraction. This could be caused by these genes being very tightly regulated to a specific tissue or developmental stage that was not present in our samples. If other tests revel that these mutations cause interesting phenotypes then we can perform expression studies that cover a more thorough representation of all plant tissues and developmental stages. However, I feel this is unwarranted until there is some indication that the information could be useful.

Two lines, *kar4* and *bim1a*, showed WT expression levels by Northern analysis but a complete lack of expression when analyzed by RT-PCR. This could be caused by the radioactively labeled probes used in Northern analysis hybridizing to homologous or paralogous genes from the same functional family (e.g. *BIM1b* in the case of *bim1a*). In contrast, the primers used in RT-PCR analysis would be sequence specific, and the sequence may not be conserved enough to allow for efficient amplification of the homologous genes. Also of interest was the line *cdc37*. Northern blots detected expression of this gene in wild type lines but not in the *cdc37* mutant; in contrast, no expression was detected in either line using RT-PCR. This may be caused by inaccurate cDNA predictions (based on genomic sequence) or undocumented splicing variations that prohibited amplification by the chosen primers. These differences may not be significant enough to effect probe hybridization, or they may be in a different part of the transcribed sequence. In short, all three of these lines (*bim1a*, *cdc37*, and *kar4*) require further analyses before the data is reasonable and clear enough for publication. However,

the time and resources required for these analyses can only be justified if the mutants are deemed interesting enough by other phenotype-based evaluations.

It is interesting that one of our lines with an obvious morphological phenotype, *ste12,* showed wild-type levels of expression under both techniques. However, the relative quantity of expression reveals little about the quality of expression—whether the RNA is full length, whether the sequence is accurately transcribed, whether the proteins is correctly modified and folded, etc. Therefore, though some form of *ste12* RNA is being produced by these lines, it is apparent that this RNA and subsequent protein is not fully functional. This may also be the case with *spc42*, although no phenotype has yet to be detected in this mutant. Alternatively, the T-DNA insertion in the *SPC42* gene is spliced out or in a region of the gene that is actually not transcribed, and thus does not affect expression levels at all. It is difficult to eliminate the latter, simpler theory without a phenotype to indicate otherwise

Phenotype Evaluation

The means of characterization of these mutant lines may seem arbitrary, but efforts were made to identify analyses that were both expedient and capable of identifying a variety of alterations in sexual reproduction. Two lines (*ste12* and *rec8*) had obvious phenotypes that affected the morphology of the entire plant. In the case of *ste12* there does not appear to be any effect on plant fertility but rather a strong effect on overall plant growth. This could be caused by alterations in a variety of systems, including developmental regulation, mitotic pathways, cell expansion and cell wall deposition, etc. However, as these mutant lines were developed under the express

purpose of alteration of plant sexual reproduction, it is questionable how much time and effort should be spent in characterizing the true cause of the *ste12* phenotype unless it can be determined that it effects fertility in some way.

In contrast, one of the most interesting aspects of the *rec8* phenotype was that it seemed to directly affect plant fertility, as indicated by shortage of virile siliques. This made homozygous *rec8* mutants ideal candidates for cytological evaluations, which did in turn help illuminate, though not completely explain, the causes of the reduced fertility. The original *rec8* gene from *S. pombe* is a well-studied subunit of the meiotic cohesion complex required for reductional chromosome segregation (Watanabe and Nurse 1999). Given this background, our initial efforts focused on analyzing meiosis and megasporogenesis in *rec8* homozygous plants. However, we detected no difference between wild-type and *rec8* megaspore development from ovule differentiation up through egg sac maturation (Figure 9). After fertilization, however, some ovules did undergo alterations in development. These egg sacs of these ovules ballooned outward while the integuments apparently degenerated. An egg cell could not be positively identified in these specimens, though there was often one, two, or four large nuclei located in the central or chalazal region. This is drastically different from the typical morphology of dying wild-type ovules. Typically, the egg sac collapses inward, and the egg and central cells persist until the egg sac is completely reabsorbed. The integuments also persist past ovule death, even to the point of still being present in the mature silique. These differences indicate that the *rec8* phenotype is not the result impaired fertilization or embryo lethality but rather alterations in the overall course of ovule development. In

addition, this phenotype did not affect all *rec8* ovules, implying there either the mutation in incompletely expressed and/or there are some unknown environmental or developmental factors at play. When this phenotype was present, it affected all the ovules in a given silique. The appearance of the style cells (elongated and acytoplasmic) indicated these siliques were pollinated, and the presence of sperm nuclei in the ovules is indicative of egg cell penetration, if not fertilization.

It was originally presumed that the large nuclei present in the *rec8* ovules were the products of mitotic divisions of either the triploid endosperm (if fertilization occurred) or the diploid central cell nucleus (if fertilization did not occur). However, these nuclei are considerable larger than those present in wild-type or unaffected *rec8* ovules at the 4-nucleus stage of endosperm development, which are also typically more spread out along the outer edge of the ovule. In addition, some of the atypical ovules have small, nucleus-like structures along their outer edges. These are roughly the same size and in the same location as nuclei in a 16- or 32-cell endosperm. If the edge nuclei are in fact the progeny of the central cell, then the large nuclei must be derived from the egg (or zygotic) cell or the sperm nuclei. The former is most likely, due to readily identifiable presence of two sperm nuclei in a small number of aberrant ovules (Figure 9F). Though in these cases the sperm nuclei have not fused with the egg or central nuclei, this cannot truly be considered an example of semigametic reproduction because of the other morphological differences that obviously affect fertilization and synkaryon formation. However, this could be evidence of some form of parthenogenic

development, as the central cell or the egg cell or perhaps both are dividing in the absence of a true fertilization event.

Lastly, our positive results with *rec8* indicate that cytological analysis in a viable and productive means of identifying when and how alterations in sexual reproduction occur. This technique can be readily applied to any other mutant lines that demonstrate a reproductive phenotype using the assays discussed below.

Quantification of Fertility

To quantify the fertility of all our mutant lines we counted the number of viable and inviable seed per silique in all available homozygous mutant lines. Lower total seed set implies a lack of female gametophyte viability, while a low fertility ratio (number of viable seeds divided by number of total seeds) implies some sort of alteration to fertilization or embryo lethality, as the pistil apparently produced mature ovules that did not progress to becoming mature seed.

The overall decrease in fertility seen in all lines could indicate that our *in silico* screen for *Arabidopsis* reproductive mutants was inordinately accurate. More likely, the T-DNA inserts or other by-products of the mutagenesis process impacted the overall fertility in the plants in ways independent from the effects of single gene mutation.

Transmission Deficiency Assay

We tested the effectiveness of this assay on two randomly selected lines (*bim1b* and *spo3*) before applying to the whole of the entire population. While gametes carrying the *spo3* mutation showed no discernable differences from wild-type gametes, *bim1b* mutants displayed a heretofore unexpected phenotype. In both male and female gametes,

the *bim1b* mutation had a deleterious effect on fitness and/or competitiveness. This was especially pronounced in the female gametes. When heterozygous plants were used as female parents in heterozygous x wild-type plants most of the progeny (72%) was homozygous wild type. This is a significant deviation from the 50% one would expect if the mutations did not affect gamete viability. There was a similar, though less pronounced effect when this cross was reversed and the heterozygous plants were used as males (64% wild type). This is interesting because there are stronger selection pressures placed on male gametes than on female gametes. Only a very small percentage of pollen cells will contribute to the next generation, and selection can be based on a variety of factors, including the timing of pollen development, how well the pollen grains transfer to receptive stigmas, rates of germination and pollen tube growth, and efficiency in finding the ovules inside the pistil. In contrast, female gametes are protected and nurtured inside many layers of maternal tissue. In theory, all egg cells should survive to produce viable embryos. However, it is possible there is some selection pressure in the short time frame between megasporogenesis and megagametogenesis. Meiosis results in the formation of four haploid daughter cells. In *Arabidopsis* (as in all plant species) only one of these four daughter cells will go on to form the egg cell; the other three will degenerate. It is possible that, through some as yet unknown mechanisms, the two megaspores that bear mutations in their *BIM1b* genes are somehow more likely to degenerate. In *Arabidopsis* megaspore survival depends on position; the chalazal most cell survives to undergo meiotic division and form the egg sac. It is possible that mutations in the *BIM1b* gene somehow affect is process and/or

the distribution of alleles among the four meiotic products.

However, while *bim1b* gametes may be less competitive that wild-type gametes, this does not mean they are completely inviable. Homozygous *bim1b* mutant plants are very fertile; in fact, our fertility assay (Figure 10) showed that *bim1b* was the mutant line that performed most similarly to wild type in both number of seed and percentage viable seed. There were no indications that this mutant had any type of reproductive phenotype, yet the mutant allele of *bim1b* displays distinctly altered segregation.

The question remains as to whether or not it would be beneficial to apply the transmission distortion assay to all of the putative reproductive mutant lines identified in this study. The lines *spo3* and *bim1b* were chosen at random; neither had shown any phenotype before this analysis. However, the results of the TDA clearly illustrate a difference between these two—*bim1b* has the potential to be an interesting mutation and should be examined more closely, while at this point *spo3* could probably be eliminated as a potential reproductive mutation. I feel these results show that the transmission distortion assay can be effectively and efficiently used to differentiate interesting reproductive mutations from uninteresting mutant lines.

It should be noted that even if certain mutant lines show no phenotype in the TDA, this does not necessarily mean they do not have any reproductive phenotype. This could be the result of various factors, including a) the putative reproductive genes we isolated in our *in silico* analysis do not effect plant reproduction at all, b) the T-DNA insertions in these genes do not effect plant reproduction in ways discernable by a transmission distortion assay, or c) transmission of the mutant alleles is affected, but not

strongly enough to be detected in populations of this size. However, there comes a point in the course of any experiment where the expenditures outweigh the rewards. At some point delimitation must be made between useful and ineffectual mutant lines, otherwise time and money will be wasted in fruitless pursuits. Applying the TDA to all mutants lines would make an effective final screening procedure to determined which lines are of interest and which, from this point forward, can be ignored.

Overall Project Success

There is not an on/off switch controlling sexuality. The relative fortuitousness of this in evolutionary terms may be debatable, but for the apomixis researchers it drastically compounds their lives. It means asexual reproduction will probably never be discovered through a simple forward genetics mutant screen or through the alteration of a single putative apomixis gene. Rather, researchers must devise ways to identify genes that control parts or aspects of sexual reproduction and alter or combine them in novel interacting ways. This will require ingenuity, creativity, and a little bit of luck. However, even as the final goal remains elusive, the process of discovering and characterizing these genes will yield insights. The more knowledge we have about them, the more we can manipulate them, and the more useful they become.

The purpose of this study was to identify potential useful alterations in plant sexual development. At the very least, discovery of the *rec8* mutant line successfully meets this goal. In addition, other lines have promising characteristics that may blossom into true, useful phenotypes given further study. Thorough characterization of all the genes identified in this study depends on concerted long-term efforts, and care needs to

be given in determining which lines to pursue and which to set aside. However, the authors feel that there is great potential here that will hopefully one day contribute to our overall understanding of sexual and asexual plant reproduction.

CONCLUSIONS

This study was an attempt to investigate a few of the diverse mechanisms plants can use to reproduce asexually. We studied a naturally occurring mutation from *Gossypium barbadense*, the *Semigamy* mutation. Mutations in the *Se* gene result in a nonrecurrent form of apomixis wherein the egg and sperm nuclei do not fuse but instead undergo separate, simultaneous mitotic divisions. In addition, we used several reverse genetics techniques to identify new potential reproductive mutations in the model species *Arabidopsis thaliana*. We had hopes that this combination of approaches would aid in determining both the mechanisms and genetic regulation underlying asexual plant reproduction.

We used a new cytology-based screening method to better analyze the mode of action of the *Se* mutation. Our results indicate that the *Se* is either expressed gametophytically or zygotically, and if the latter the mutant allele acts recessive to the wild-type. We also showed that the mutations is expressed in >90% of homozygous ovules. This stands in direct contradiction to previous reports, which characterized the *Se* as dominant to wild-type an incompletely expressed. This contradiction is best explained through a comparison of the different phenotypes under evaluation: previous studies have relied on the frequency of haploid and/or chimeric progeny, while our methods score directly for the presence or absence of nuclear fusion. The former technique can be easily obscured by the effects of the first mitotic division and/or any factors that differentially imperil haploid tissues, e.g. inbreeding depression, which would explain apparent incomplete expression of the phenotype. In contrast, our

screening method allows for the quick and reliable screening for the *Se* phenotype, and even permits the differentiation of heterozygotes from homozygotes. This information will hopefully aid in generating a better understanding of the mechanism underlying the *Se* phenotype and, hopefully, other forms of apomixis.

We also devised an experiment to localize the *Se* gene to a particular chromosomal segment through the creation of backcrossed aneuploid *hirsutum* x homozygous *Se* mutant *barbadense* populations. Individuals form these populations were genotyped by phonological analysis, and we attempted to identify a population that did not segregate for the *Se* mutation. Unfortunately, all of our populations segregated, indicating the *Se* gene is not located on any of the chromosomal segments included in our study. However, this study did provide us with a large collection of individual plants that have been scored for *Semigamy* expression. DNA collected from these individuals can be used in a molecular-marker based mapping experiment that should localize the *Se* gene to a particular chromosomal arm or linkage group. In addition, our results have eliminate >50% of the cotton genome as a potential location for the *Se* locus, which should aid in future mapping efforts. Linkage to an easily selectable genetic marker would greatly increase the utility of the *Se* mutation, and hopefully these efforts will contribute to the overall search for such a marker.

Lastly, our reverse genetics studies in *Arabidopsis* yielded several putative mutants of interest that hold great potential for further study. This study started with over 200 genes of interest from a variety of species. Each of these was compared against the known database of *Arabidopsis* proteins in an effort to identify evolutionarily

conserved homologs. We then obtained lines carrying T-DNA insertional mutations in these potential homologues screened them for various phenotypes. Some mutant lines had obvious physical phenotypes (e.g. *ste12* and *rec8*), while others only showed the effects of the mutation under detailed analysis (e.g. *bim1b*). Though some analyses were less helpful than others (e.g. our fertility assay showed very little differences between the mutant lines), we believe the segregation distortion assay (TDA) will effectively screen the reproductive mutants from the non-reproductive ones. We performed this assay on two of our putative mutant lines as a proof of concept. In the line *bim1b,* gametes carrying the mutant allele were less viable than their wild-type counterparts. However, the *spo3* mutation had little effect on gamete viability. In summary, though not all the lines identified through our reverse genetics approach will be useful or interesting to the study of plant reproduction, we have uncovered several new mutants that could help elucidate the diverse mechanisms involved in sexual and asexual plant reproduction.

The overall goal of this project was to make a significant contribution to our understanding of the mechanisms underlying plant reproduction n all its various forms. A thorough understanding of plant reproduction will better permit its manipulation, both in the lab and in the field. Ideally, this and complementary research in labs all over the world will one day enable us to create useful asexual crop species for the benefit of farmers and producers everywhere.

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APPENDIX A

Table 9. All the Genes Identified as Being "of Interest" in the Initial *in silico* Screening

Gene Name	Phenotype	Organism	Source
18S ribosomal protein AF093506	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
60S ribosomal protein L12 U93168	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
adenosy-homocysteinase L36119	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
ady1	forms abnormal dyads after MI and MII	*S. cerevisiae*	Rabitsch et al. 2001
ady2	forms abnormal dyads after MI and MII	*S. cerevisiae*	Rabitsch et al. 2001
ady3	forms abnormal dyads after MI and MII	*S. cerevisiae*	Rabitsch et al. 2001
ady4	forms abnormal dyads and monads after MI and MII	*S. cerevisiae*	Rabitsch et al. 2001
afd	"substitution of first meiotic division by mitosis;" plants are completely male and female sterile	*Z. Mays*	Golubovskaya 1989
alanin:glyoxylate aminotransferase AGT2 AF166351	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
Alcohol dehydrogenase III L-chain P81601	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001

Table 9. Continued

Gene Name	Phenotype	Organism	Source
18S ribosomal protein AF093506	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
60S ribosomal protein L12 U93168	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
adenosy-homocysteinase L36119	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
ady1	forms abnormal dyads after MI and MII	*S. cerevisiae*	Rabitsch et al. 2001
ady2	forms abnormal dyads after MI and MII	*S. cerevisiae*	Rabitsch et al. 2001
ady3	forms abnormal dyads after MI and MII	*S. cerevisiae*	Rabitsch et al. 2001
ady4	forms abnormal dyads and monads after MI and MII	*S. cerevisiae*	Rabitsch et al. 2001
afd	"substitution of first meiotic division by mitosis;" plants are completely male and female sterile	*Z. Mays*	Golubovskaya 1989
alanin:glyoxylate aminotransferase AGT2 AF166351	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
Alcohol dehydrogenase III L-chain P81601	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
Allene oxide synthase AJ271093	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
Alpha-1 tubulin AA063914	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001

Table 9. Continued

Gene Name	Phenotype	Organism	Source
Alpha-1 tubulin BF598276	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
Alpha-N-acetylglucosaminidase Y18209	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
am2(ameiotic-2)	mutant lacks first meiotic division	*Z. Mays*	Curtis and Doyle 1991
ama1	Double mutant with *spo13* produces diploid progeny. *Ama1* blocks meiosis I (on its own this halts meiosis completely), when combined with *spo13* meiosis is completed with only one division.	*S. cerevisiae*	Cooper et al. 2000
ameiotic (am1)	Instead of meiosis there are two to three synchronous mitoses and subsequent chromatin degradation. (= produces high numbers of diploids)	*Z. Mays*	Golubovskaya 1989
apg1	protein involved in induction of autophagy; mutant does not undergo meiosis	*S. cerevisiae*	Briza et al. 2002
apg14	required for autophagy; mutant does not undergo meiosis	*S. cerevisiae*	Briza et al. 2002
apg9	deletion mutants undergo both meiotic divisions but fail to form spores or asci	*S. cerevisiae*	Rabitsch et al. 2001
arl2	homologue of *cin4*	*H. Sapiens*	Bhamidipati et al. 2000

Table 9. Continued

Gene Name	Phenotype	Organism	Source
asd1-1	Mutants self-diploidize (alternative self-diploidization) which could be an interesting phenotype if it were expressed only in the gametes (otherwise get 4n plants)	*S. cerevisiae*	Ono et a. 1990
aut2	protein required for autophagy; mutant does not undergo meiosis	*S. cerevisiae*	Briza et al. 2002
beta-1 tubulin M12296	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
bik1	microtubule associated protein required for karyogamy	*S. cerevisiae*	Kurihara et al. 1994
bim1	karyogamy mutation	*S. cerevisiae*	Schwartz et al. 1997
BiP	Human homologue of *kar2*	*H. Sapiens*	Rose et al. 1989
B-Tubulin mRNA	a candidate gene for apomeiosis (highly expressed in the apomictic mutant)	*M. sativa*	Barcaccia et al. 2001
bud32	possibly involved in polar bud site selection in diploid cells; mutant does not undergo meiosis	*S. cerevisiae*	Briza et al. 2002
byr1	interacts somehow with *spk1* (probably upstream); mutants are sterile	*S. pombe*	Gotoh et al. 1993
byr2	interacts somehow with *spk1*; mutants are sterile	*S. pombe*	Gotoh et al. 1993
c(3)G (17 and 68)	mutants show altered disjunction in all chromosomes in female only	*D. melanogaster*	Baker and Hall 1976

Table 9. Continued

Gene Name	Phenotype	Organism	Source
ca (NM_057303)	causes abnormal chromosome behavior and massive chromosome non-disjunction	*D. melanogaster*	Baker and Hall 1976
cak1	mutants show substantial defects in either MI or MII. However, mutants also show significant delay in pre-meiotic DNA synthesis	*S. cerevisiae*	Schaber et al. 2002
car1	Vital for peroxisome construction, but fungal mutants do not undergo karyogamy	*Podospora anserine*	Berteaux-Lecellier et al. 1995
ccz1	involved in vesicular transport and vacuolar assembly; mutant does not undergo meiosis	*S. cerevisiae*	Briza et al. 2002
cdc02	produces two-spored asci	*S. pombe*	Nakaseko et al. 1984
cdc04	cell division cycle gene, mutants are karyogamy deficient	*S. cerevisiae*	Dutcher and Hartwell 1982
cdc05	Usually fatal, but survivors produce 2-spored, diploid asci	*S. pombe*	Nakaseko et al. 1984
cdc07	protein kinase that stimulates entry into MII	*S. cerevisiae*	Nakamura et al. 2002
cdc09	dispensable for intragenic recombination, but required for haploidization and spores	*S. cerevisiae*	Kupiec et al. 1997
cdc14	Mutations arise in both mitosis and meiosis, so this mutation is usually fatal. However, mutants that do survive produce two diploid spores in their asci.	*S. cerevisiae*	Schild and Byers 1980

Table 9. Continued

Gene Name	Phenotype	Organism	Source
cdc25	responsible for the choice between meiosis and mitosis	*S. cerevisiae*	Golubovskaya 1989
cdc26	implicated in controlling meiotic divisions	*S. cerevisiae*	Chu et al. 1994
cdc28	cell division cycle gene. Mutants are karyogamy deficient and, under certain conditions, arrest with reductional dyads after failure to undergo MII	*S. cerevisiae*	Dutcher and Hartwell 1982 & Kupiec et al. 1997
cdc31	forms reductional dyads due to lack of SPB duplication in mitosis and MII; interacts with Kar1	*S. cerevisiae*	Kupiec et al. 1997
cdc34	cell division cycle gene, mutants are karyogamy deficient	*S. cerevisiae*	Dutcher and Hartwell 1982
cdc35	with *cdc25*, responsible for the choice between meiosis and mitosis	*S. cerevisiae*	Golubovskaya 1989
cdc37	cell division cycle gene, mutants are karyogamy deficient	*S. cerevisiae*	Dutcher and Hartwell 1982
cdc48	Cell cycle protein required for karyogamy and peroxisome function	*S. cerevisiae*	Latterich et al. 1995
CDEII	required specifically for MI disjunction	*S. cerevisiae*	Kupiec et al. 1997
Chlorophyll a/b-binding protein type II X14506	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
Chlorophyll a/b-binding protein LCHPII S73603	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001

Table 9. Continued

Gene Name	Phenotype	Organism	Source
cik1	interacts with *Kar3*	*S. cerevisiae*	Shanks et al. 2001
cin1	MT associated protein	*S. cerevisiae*	Hoyt et al. 1990
cin2	MT associated protein	*S. cerevisiae*	Hoyt et al. 1990
cin4	MT associated protein	*S. cerevisiae*	Hoyt et al. 1990
cin8	another *KAR3* subfamily member	*S. cerevisiae*	Hoyt et al. 1990
c-kit	proto-oncogene; when overexpressed females are parthenogenic	*M. musculus*	Sette et al. 1997
clb1	possibly involved in sister chromatid segregation and MII	*S. cerevisiae*	Kamieniecki et al. 2000
clb4	possibly involved in sister chromatid segregation and MII	*S. cerevisiae*	Kamieniecki et al. 2000
c-mos	Mouse proto-oncogene; mutation causes spontaneous parthenogenic development	*M. musculus*	Colledge et al. 1994
cnm67	component of the spindle pole body; mutant does not undergo meiosis	*S. cerevisiae*	Briza et al. 2002
ctf19	mutation produces massive meiotic chromosome mis-segregation	*S. cerevisiae*	Rabitsch et al. 2001
Cytoskeletal keratin-like AW767031	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001

Table 9. Continued

Gene Name	Phenotype	Organism	Source
dbf4	stimulates *cdc07* (which in turn promotes entry in MII)	*S. cerevisiae*	Nakamura et al. 2002
desynaptic (dsy)	incomplete pairing of homologous chromosomes	*Z. Mays*	Curtis and Doyle 1991
desynaptic (dy)	desynapsis of chromosomes	*Z. Mays*	Golubovskaya 1989
dhc1	When combined with *klp2* or *pkl1* results in a karyogamy defect	*S. pombe*	Troxell et al. 2001.
divergent spindle (dv)	regular divisions; irregular spindle formation	*Z. mays*	Curtis and Doyle 1991
dlc1	mutation has karyogamy and sporulation defects, especially when combined with *dhc1*	*S. cerevisiae*	Miki et al. 2002
dyad	Mutants arrest after a single division	*A. thaliana*	Bhatt et al. 2001
Early light inducible protein ELIP U82810	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
Early light inducible protein-precursor CAA29399	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
elongate (el)	abnormalities of second meiotic division; variable penetrance and effects, but sometimes cells are not completely divided	*Z. mays*	Rhoades and Dempsey 1966
eq	affects MII division, however data is inconclusive and hazy	*D. melanogaster*	Baker and Hall 1976

Table 9. Continued

Gene Name	Phenotype	Organism	Source
Ethylene-regulated protein ER6 AF096262	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
fc	failure of cytokinesis (SDR); is followed by nuclear fusion	*S. tuberosum*	Peloquin et al. 1999
female-sterile (fs)	mutants are sterile, but produce many aposporous embryo sacs	*P. americanum*	Hanna and Powell 1974
ff16	mutants show a lack of meiosis I cytokinesis	*D. melanogaster*	Fedorova et al. 2001
Flower bud CDNA clone cTOD6P5 BE353911	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
frt1	mutation is able to induce development when introduced into sterile strains. Mechanism is unknown, but could be interesting	*C. cinereus*	Kamada 2002
fus1	required for sperm/egg cell fusion	*S. cerevisiae*	Kurihara et al. 1994
fus2	effects karyogamy and cell fusion	*S. cerevisiae*	Elion et al. 1995
fus3	required for sperm/egg cell fusion	*S. cerevisiae*	Kurihara et al. 1994
fus5 (AXL1)	required for sperm/egg cell fusion	*S. cerevisiae*	Kurihara et al. 1994
fus6 (SPA2)	required for sperm/egg cell fusion	*S. cerevisiae*	Kurihara et al. 1994
fus7 (RVS161)	required for sperm/egg cell fusion	*S. cerevisiae*	Kurihara et al. 1994

Table 9. Continued

Gene Name	Phenotype	Organism	Source
fzr1	Though mutants divide normally, they form only two spores (a cytokinesis mutant?)	*S. pombe*	Asakawa et al. 2001
gip1	deletion mutants undergo both meiotic divisions but fail to form spores or asci	*S. cerevisiae*	Rabitsch et al. 2001
gld1	worms with this mutation cannot enter meiosis, instead, germline cells continue to undergo mitosis	*C. elegans*	Kadyk and Kimble 1998
gld2	worms with this mutation cannot enter meiosis, instead, germline cells continue to undergo meiosis	*C. elegans*	Kadyk and Kimble 1998
hfd1	mutants form unstable spore walls and often form dyads containing non-sister spores	*S. cerevisiae*	Kupiec et al. 1997
hsk1	another possible homologue of *spo4*	*S. pombe*	Nakamura et al. 2002
idole-3-acetate beta-g-transferase BAA93039	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
jem1	karyogamy mutant	*S. cerevisiae*	Nishikawa and Endo 1997
jp	mutant produces diploid sperm	*M. sativa*	Mariani et al. 2000
kar1	located in spindle pole body (SPB)	*S. cerevisiae*	Conde and Fink 1976
kar2	homologue of human *BiP*; a chaperone protein	*S. cerevisiae*	Rose et al. 1989
kar3	kinesin heavy chain related protein	*S. cerevisiae*	Meluh and Rose 1990

Table 9. Continued

Gene Name	Phenotype	Organism	Source
kar4	regulates *KAR3* and *Cik1*	*S. cerevisiae*	Kurihara et al. 1994
kar5	membrane protein involved solely in karyogamy	*S. cerevisiae*	Beh et al. 1997
kar7	allelic to *sec71* and *sec66*	*S. cerevisiae*	Brizzion et al. 1999
kar8	Alternative name for *JEM1*	*S. cerevisiae*	Brizzion et al. 1999
kar9	responsible for nuclear movement	*S. cerevisiae*	Miller and Rose 1998
kel2	involved in membrane fusions	*S. cerevisiae*	Tzung et al. 2001
kex2 (1)	dispensable to premeiotic DNA synthesis and meiotic recombination but partially required for MI and/or MII. Mutants fail in the final stages of sporulation.	*S. cerevisiae*	Leibowitz and Wickner 1976 & Kupiec et al. 1997
kip1	another *KAR3* subfamily protein	*S. cerevisiae*	Saunders and Hoyt 1992
klp2	A member of the *KAR3* subfamily in yeast	*S. pombe*	Troxell et al. 2001
klp5	kinesin motor protein that promote microtubule disassembly. Mutants show disrupted chromosome segregation. Phenotype is exasperated in the presence of *klp5* and *klp6* double mutants	*S. pombe*	West et al. 2001
klp6	kinesin motor protein that promote microtubule disassembly. Mutants show disrupted chromosome segregation. Phenotype is exasperated in the presence of *klp5* and *klp6* double mutants	*S. pombe*	West et al. 2001

Table 9. Continued

Gene Name	Phenotype	Organism	Source
klpA	A fungal member of the *Kar3* subfamily	*A. nidulans*	O'Connell et al. 1993
kms1	karyogamy defective	*S. pombe*	Shimanuki et al. 1997
LEA protein EMB8 TO8400	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
leptotene or "L-protein"	"considered to be responsible for the irreversible commitment of cells to meiosis after entering the premeiotic S phase"	*Z. Mays*	Golubovskaya 1989
mal3	May act similarly to *Bim3*; associated with microtubule stability	*S. pombe*	Asakawa et al. 2005
mam1	essential for proper orientation of the sister kinetochores in MI	*S. cerevisiae*	Rabitsch et al. 2001
mei1	meiotic mutant	*M. musculus*	Hunt and Hassold 2002
mei-160	mutants show altered disjunction in all chromosomes in female only	*D. melanogaster*	Baker and Hall 1976
mei-251	mutants show altered disjunction in all chromosomes in female only	*D. melanogaster*	Baker and Hall 1976
mei-352	mutants show altered disjunction in all chromosomes in female only	*D. melanogaster*	Baker and Hall 1976
mei-38 (2)	mutants show altered disjunction in all chromosomes in female only	*D. melanogaster*	Baker and Hall 1976

Table 9. Continued

Gene Name	Phenotype	Organism	Source
mei-41, mei-195	mutants show altered disjunction in all chromosomes in female only	*D. melanogaster*	Baker and Hall 1976
mei5	mutants have no nuclear divisions	*S. cerevisiae*	Rabitsch et al. 2001
mei9 (NA:U27181)	mutation causes non-disjunction of various chromosomes; homologue of yeast *rad1*	*D. melanogaster*	Baker and Hall 1976
mei-99 (2)	mutants show altered disjunction in all chromosomes in female only	*D. melanogaster*	Baker and Hall 1976
mei-B	mutants show altered disjunction in all chromosomes in female only	*D. melanogaster*	Baker and Hall 1976
mei-O81	mutation causes non-disjunction (low expression)	*D. melanogaster*	Baker and Hall 1976
mei-S282	mutants show altered disjunction in all chromosomes in female only	*D. melanogaster*	Baker and Hall 1976
mei-S332	mutants are dysfunctional in both MI and MII, very high levels of nondisjunction	*D. melanogaster*	Schild and Byers 1980
mei-S51	mutants show altered disjunction in all chromosomes in female only	*D. melanogaster*	Baker and Hall 1976
mes1	mutants accumulate two-nucleated zygotes that are incapable of sporulation	*S. pombe*	Nakaseko et al. 1984
mes2	mutants accumulate two-nucleated zygotes that are incapable of sporulation	*S. pombe*	Nakaseko et al. 1984

Table 9. Continued

Gene Name	Phenotype	Organism	Source
mfr1	coordinates nuclear divisions and yeast cell wall formation. Mutants undergo regular meiosis but delayed spore formation.	*S. cerevisiae*	Blanco et al. 2001
mlh1	meiotic mutant	*M. musculus*	Hunt and Hassold 2002
mnd1	required for meiotic nuclear division; probably involved in sporulation initiation	*S. cerevisiae*	Briza et al. 2002
mnd2	mutants have no nuclear divisions	*S. cerevisiae*	Rabitsch et al. 2001
Mob-like protein T40465	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
mon1	mutant does not undergo meiosis	*S. cerevisiae*	Briza et al. 2002
mpc54	deletion mutants undergo both meiotic divisions but fail to form spores or asci	*S. cerevisiae*	Rabitsch et al. 2001
mpc70	deletion mutants undergo both meiotic divisions but fail to form spores or asci	*S. cerevisiae*	Rabitsch et al. 2001
mps2	required for proper SPB modification in MII; mutants produce each MI genome in reductional dyads.	*S. cerevisiae*	Kupiec et al. 1997
mrps-18	mitochondrial ribosomal protein; mutant does not undergo meiosis	*S. cerevisiae*	Briza et al. 2002
msd	currently uncharacterized meiotic mutant (lack of megasporogenesis)	*A. thaliana*	Bhatt et al. 2001

Table 9. Continued

Gene Name	Phenotype	Organism	Source
mvh	meiotic mutant	*M. musculus*	Hunt and Hassold 2002
ncd	related to *kar3* and *klpA*	*D. melanogaster*	O'Connell et al. 1993
nda5	produced two-spored asci	*S. pombe*	Nakaseko et al. 1984
ndc1	required for MII	*S. cerevisiae*	Kupiec et al. 1997
Ndt80	required for expression of middle/late meiotic genes (meiotic divisions and sporulation)	*S. cerevisiae*	Tzung et al. 2001
nmr1	no meiosis, arrests before DNA synthesis (=> 2n cells)	*S. cerevisiae*	Rabitsch et al. 2001
nod	in mutants recombination is unaffected and non-exchange homologues almost always fail to disjoin	*D. melanogaster*	Baker and Hall 1976
Nod factor binding LNP AF156782	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
Non-functional folate binding protein AAD00154	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
num1	nuclear movement protein. Mutants are generally polyploid including vegetative cells and spores.	*S. cerevisiae*	Kormanec et al. 1991
ord	mutants are dysfunctional in both MI and MII	*D. melanogaster*	Schild and Byers 1980
os	"omission of the second division" (SDR); the predominant mechanism of 2n-egg formation in potato	*S. tuberosum*	Peloquin et al. 1999

Table 9. Continued

Gene Name	Phenotype	Organism	Source
Ovary cDNA clone cLED32K8 AI898230	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
p97	homologue of *cdc48*	*R. norvegicus*	Meyer et al. 1998
paf1	homologue of *car1*	*H. Sapiens*	Berteaux-Lecellier et al. 1995
pam1	unspecific meiosis mutation; however, produces multinucleate and polyploid cells	*Z. Mays*	Golubovskaya 1989
pam2	unspecific meiosis mutation; however, produces multinucleate and polyploid cells	*Z. Mays*	Golubovskaya 1989
parallel spindles (ps)	homozygous mutants produce diploid pollen as a result of First Division Restoration (FDR)	*S. phureja*	Buso et al. 1999
Pca-1	a differentially expressed cDNA found only in both apomictic and sexual ovaries	*P. ciliare*	Vielle-Calzada et al. 1996
Pca-2	a differentially expressed cDNA found only in apomictic ovaries	*P. ciliare*	Vielle-Calzada et al. 1996
Pca-3	a differentially expressed cDNA found only in apomictic ovaries	*P. ciliare*	Vielle-Calzada et al. 1996
Pcs-2	a differentially expressed cDNA found only in sexual ovaries	*P. ciliare*	Vielle-Calzada et al. 1996
pep4	required for haploidization and sporulation	*S. cerevisiae*	Kupiec et al. 1997

Table 9. Continued

Gene Name	Phenotype	Organism	Source
per6	homologue of *car1*	*P. pastoris*	Waterham et al. 1996
pex19	mutant undergoes WT meiosis; protein is involved in peroxisome biosynthesis (personal curiosity)	*S. cerevisiae*	Briza et al. 2002
pex2	homolog of *car1*	*S. cerevisiae*	Titorenko and Rachubinski 2001
phosphate-starved leaf cDNA clone BF638159	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
Pistil specific protein sts15 T07677	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
pkl1	member of the "*KAR3* subfamily" in yeast	*S. pombe*	Troxell et al. 2001
pms2	meiotic mutant	*M. musculus*	Hunt and Hassold 2002
polymitotic (po)	mutant shows "supernumerary second divisions"	*Z. Mays*	Curtis and Doyle 1991
prm1	involved in membrane fusion during yeast mating	*S. cerevisiae*	Heiman and Walter 2000
Putative hydrolast TzH20.280 AL162508	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
Putative protein F22K18.140 T05568	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
rad1	yeast cells containing mutated *rad1* and *rad8* show high levels of diploid spore formation.	*S. cerevisiae*	Dowling et al. 1985

Table 9. Continued

Gene Name	Phenotype	Organism	Source
rad6	required for meiotic gene conversion and haploidization	*S. cerevisiae*	Kupiec et al. 1997
rad8	yeast cells containing mutated *rad1* and *rad8* show high levels of diploid spore formation.	*S. cerevisiae*	Dowling et al. 1985
ras1	interacts somehow with *spk1*; mutants are sterile	*S. pombe*	Gotoh et al. 1993
RD	mutants affected in meiotic drive	*D. melanogaster*	Baker and Hall 1976
rec114	homologue of *rec7*, a promising meiotic gene (produces two haploid spores, failure of first division and non-disjunction)	*S. cerevisiae*	Molnar et al. 2001
rec15	mutations have been found in some experiments ("unpublished results") to produce high frequencies of dyads	*S. cerevisiae*	Molnar et al. 2001
rec7	an early meiotic protein. Mutants produce two haploid spores. Phenotype is probably the results ofskipping the first meiotic division. Non-disjunction is fairly common. A probable homologue of *S. cerevisiae rec114*	*S. pombe*	Molnar et al. 2001
rec8	mutants undergo equational instead of reductional division at Meiosis I (sister chromatids split apart early). This leads to formation of asci with two diploid spores, but in low numbers.	*S. pombe*	Watanabe and Nurse 1999
rim4	no meiosis, arrests before DNA synthesis (=> 2n cells)	*S. cerevisiae*	Rabitsch et al. 2001

Table 9. Continued

Gene Name	Phenotype	Organism	Source
rsm23	protein required for respiration and mitochondrial maintenance; mutant does not undergo meiosis	*S. cerevisiae*	Briza et al. 2002
sag1	provided for tight cell-cell adhesion during mating (a long shot protein, it may be involved in cell-cell fusions of sperm and egg)	*S. cerevisiae*	Tzung et al. 2001
sap	mutants undergo only one meiotic division, then arrest	*A. thaliana*	Bhatt et al. 2001
s-beta-1 tubulin AA34009	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
SD	mutants affected in meiotic drive	*D. melanogaster*	Baker and Hall 1976
sec63	moderate karyogamy effects	*S. cerevisiae*	Brizzio et al. 1999
sec72	moderate karyogamy effects	*S. cerevisiae*	Brizzio et al. 1999
Seed lipoxygenase LOX2 AW395627	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
sep1	karyogamy mutant with increased chromosome loss, a karyogamy defect, impaired spindle pole body separation, and defective nuclear migration (AKA *kem1, xrn1, dst1*, and *rar5*)	*S. cerevisiae*	Interthal et al. 1995
slk19	acts similarly to *kar3* in *Saccharomyces cerevisiae*; possibly share repetitive function; additionally, *slk19* mutants skip the first division of meiosis and produce about 50% viable diploid spores.	*S. cerevisiae*	Zeng et al. 1999 & Zeng and Saunders 2000

Table 9. Continued

Gene Name	Phenotype	Organism	Source
sma1	involved in prospore membrane formation	*S. cerevisiae*	Rabitsch et al. 2001
sma2	involved in prospore membrane formation	*S. cerevisiae*	Rabitsch et al. 2001
snf1	in yeast is responsible for the initiation of meiosis. Mutants do not enter meiosis	*S. cerevisiae*	Purnapatre et al. 2002
spc42	mutation results in ~40% of offspring in two haploid cells in the asci. WT protein is essential part of spindle pole body.	*S. cerevisiae*	Ishihara et al. 2001
spc72	interacts with *kar1*, mutation causes moderate karyogamy effects	*S. cerevisiae*	Brizzio et al. 1999 & Pereira et al. 1999
spe2	required for haploidization and sporulation	*S. cerevisiae*	Kupiec et al. 1997
spk1	sporulation gene; mutants do not sporulate; mechanism unknown	*S. pombe*	Gotoh et al. 1993
spo11	a recombination mutant that fails to produce viable spores	*S. cerevisiae*	Klapholz et al. 1985
spo12	mutants produce two diploid spores; results from a failure of second division in meiosis	*S. cerevisiae*	Klapholz and Esposito 1980
spo13	like *spo12* (but both are independent) produces two diploid asci where there should be four haploid asci. Impaired second division of meiosis.	*S. cerevisiae*	Klapholz and Esposito 1980
spo14	required for commitment to meiosis, MII, and spores. Most arrest at the bi-nucleate stage	*S. cerevisiae*	Kupiec et al. 1997

Table 9. Continued

Gene Name	Phenotype	Organism	Source
spo15	mutants complete the initial meiosis stages, but are arrested in the division stages (both of them). This might not be helpful, but it could be interesting	*S. cerevisiae*	Yeh et al. 1991
spo16	required for recombination and haploidization	*S. cerevisiae*	Kupiec et al. 1997
spo21	mutant forms immature spores; protein required for sporulation	*S. cerevisiae*	Briza et al. 2002
spo3	produces asci with one or two randomly packaged haploid spores	*S. cerevisiae*	Kupiec et al. 1997
spo4	homologue of *cdc07*	*S. pombe*	Nakamura et al. 2002
spo6	mutants undergo first meiotic division, but arrest at second. Spores are usually non-viable (could be a problem). In few instances where cells do divide, daughter cells remain closely associated.	*S. pombe*	Nakamura et al. 2000
spo70	deletion mutants undergo both meiotic divisions but fail to form spores or asci	*S. cerevisiae*	Rabitsch et al. 2001
spo71	deletion mutants undergo both meiotic divisions but fail to form spores or asci	*S. cerevisiae*	Rabitsch et al. 2001
spo72	mutant does not undergo meiosis	*S. cerevisiae*	Briza et al. 2002
spo73	deletion mutants undergo both meiotic divisions but fail to form spores or asci	*S. cerevisiae*	Rabitsch et al. 2001

Table 9. Continued

Gene Name	Phenotype	Organism	Source
spo74	deletion mutants undergo both meiotic divisions but fail to form spores or asci	*S. cerevisiae*	Rabitsch et al. 2001
spo75	deletion mutants undergo both meiotic divisions but fail to form spores or asci	*S. cerevisiae*	Rabitsch et al. 2001
spo77	deletion mutants undergo both meiotic divisions but fail to form spores or asci	*S. cerevisiae*	Rabitsch et al. 2001
spot16	required for MII and spores	*S. cerevisiae*	Kupiec et al. 1997
spot17	required for MII and spores	*S. cerevisiae*	Kupiec et al. 1997
spot18	required for MII and spores	*S. cerevisiae*	Kupiec et al. 1997
srs2	required for transition from MI to MII	*S. cerevisiae*	Kupiec et al. 1997
ssp1	deletion mutants undergo both meiotic divisions but fail to form spores or asci	*S. cerevisiae*	Rabitsch et al. 2001
ssp2	deletion mutants undergo both meiotic divisions but fail to form spores or asci	*S. cerevisiae*	Rabitsch et al. 2001
ste12	required for cell cycle arrest and karyogamy	*S. cerevisiae*	Tzung et al. 2001
stubby-head	mutants are facultative apomicts and produce multiple aposporous embryo sacs	*P. americanum*	Hanna and Powell 1973
Sugar transporter-integral membrane protein U64902	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001

Table 9. Continued

Gene Name	Phenotype	Organism	Source
swi1	hypothesized to control the meiotic/mitotic control. Mutants undergo an extra mitotic division before meiosis	*A. thaliana*	Bhatt et al. 2001
synaptic-3 (sy-3)	when combined with the *ps* mutant, produces haploid pollen by FDR without crossover events	*S. phureja*	Buso et al. 1999
tht1	gene required for karyogamy	*S. pombe*	Tange et al. 1998
tub2	yeast beta-tubulin gene, required for karyogamy	*S. cerevisiae*	Kurihara et al. 1994
tws1	mutants undergo normal mitosis but produce two diploid spores as a product of meiosis	*S. pombe*	Nakaseko et al. 1984
Ubiquitin -like protein AJ270957	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
unknown protein AC063914	homologous match to an EST that is differentially expressed in apomictic and sexual flowers	*M. sativa*	Barcaccia et al. 2001
vam6	involved in the last step of vacuolar assembly; mutant does not undergo meiosis	*S. cerevisiae*	Briza et al. 2002
variable (va1)	irregular cytokinesis	*Z. Mays*	Golubovskaya 1989
variable sterile-2 (va2)	MI and II followed by irregular cytokinesis; "cell plate either failed to form or did not form completely"	*Z. Mays*	Curtis and Doyle 1991
vik1	vegetative interacter with *kar3*	*S. cerevisiae*	Manning et al. 1999

Table 9. Continued

Gene Name	Phenotype	Organism	Source
ycr233w	mutation produces massive meiotic chromosome mis-segregation	*S. cerevisiae*	Rabitsch et al. 2001
ydr065w	no meiosis, arrests before DNA synthesis (=> 2n cells)	*S. cerevisiae*	Rabitsch et al. 2001
ygl064c	member of the DEAD-box family; mutant does not undergo meiosis	*S. cerevisiae*	Briza et al. 2002
ygr226c	mutants produce no asci; protein required for proper meiosis and sporulation	*S. cerevisiae*	Briza et al. 2002
yll033w	mutant does not undergo meiosis	*S. cerevisiae*	Briza et al. 2002
ynl081c	putative mitochondrial ribosomal protein; mutant does not undergo meiosis	*S. cerevisiae*	Briza et al. 2002
ynl177c	possibly involved in mitochondrial translation; mutant does not undergo meiosis	*S. cerevisiae*	Briza et al. 2002

VITA

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